Study of the reactivity of a monoclonal antibody that recognises human marginal zone B cells and a subset of germinal centre cells in tissue sections

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Thesis submitted for MPhil degree in immunology

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Abstract:

Marginal Zone (MZ) cells are a subset of B cells. They reside the marginal zone outside the mantle zone of the spleen and circulate the blood in humans. They have also been identified in the crypt epithelium of tonsil, the sub-capsular sinus in lymph node and in the dome area of Peyer's patches in gut-associated lymphoid tissue of human intestine. A monoclonal antibody (4D12) that recognises an antigen on MZ B cells and a subset of GC cells in tissue sections has been described previously but this has not been investigated in detail and features of the 4D12 antigen and the lymphocytes that express it are unknown. The re-establishment and culture of the 4D12 MoAb secreting clone after a long-term storage is described. A method to analyse the distribution of 4D12 antigen on subsets of B cells was devised. Subsets expressing 4D12 antigen included CD27+IgM+IgD+ cells, CD27+IgM+IgD- cells, transitional B cells and plasmablasts. Lower expression by mature naïve and class switched memory B cells was observed. 4D12 MoAb tended to recognise a greater proportion of CD27+IgM+IgD+ cells and CD27+IgM+IgD- cells in spleen compared to tonsil and blood. Intracellular staining showed that the 4D12 antigen is proportionally more presented in the cytoplasm than the cell surface and that cytoplasmic 4D12 antigen expression is not restricted to B cells. The identity of the antigen was sought by western blotting and mass spectrometry (by collaborators) and the output of the mass spectrometry was provided for this study. Mass spectrometry data was analysed here and a list of candidates for the identity of 4D12 antigen were selected and tested by flow cytometry.

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Chapter 1:
Introduction

1.1 B cell development

This is the study of a monoclonal antibody reported to recognise B cells in tissue sections. B cells originate in the bone marrow. They mature to express a vast array of different B cell receptors (BCR's) that have the ability to bind to almost any antigenic shape and initiate an antibody response. This diversity is a consequence of the recombination of variable (V) diversity (D) and joining (J) genes of the heavy chain and V and J genes of the light chain during B cell development that enable the formation of diverse functional membrane immunoglobulin (Ig) in mature B cells [1, 2].

1.2 B cell activation

The B cell immune response against pathogens depends on the recognition of antigens via two distinctive pathways. First, the T-dependent (TD) response for which T cell help is required. Naïve B cells in spleen or lymph nodes encounter antigens via their BCR causing activation of B cells. Antigen is then engulfed via endocytosis and processed into peptides. In this case, B cells act as antigen presenting cells (APC) and present peptide by class two major histocompatibility complex (MHC II) and move toward B cell zone border with the T cell zone. Previously activated CD4 T cells up-regulate CXC-chemokine receptor 5 (CXCR5) having encountered peptides presented by dendritic cells in the context of MHC II. They then move towards B cell zone. At the B cell - T cell border, CD4+ T cells recognise MHC II-bound peptide on B cells via the T cell receptor (TCR). T cells engage CD40 to CD40L and cytokine secretion to B cells providing a co-stimulatory help to fully activate B cells. Fully activated B cells then move to the dark zone in the germinal centre (GC) as centroblasts and undergo clonal expansion and somatic hypermutation of the V(D)J genes catalysed by activation-induced cytidine deaminase (AID) that increases repertoire variability. Subsequently, B cells move into the light zone of GC as centrocytes to undergo affinity maturation by selection of high affinity variants. Centrocytes compete for antigens retained by follicular dendritic cells and highest affinity cells that compete successfully for antigen present peptides to acquire help from CD4

T cells. Low affinity cells die by apoptosis. Ultimately, this leads to differentiation to plasma cells secreting high affinity antibodies or memory B cells (**Figure 1.1**) [3, 4, 5, 6].

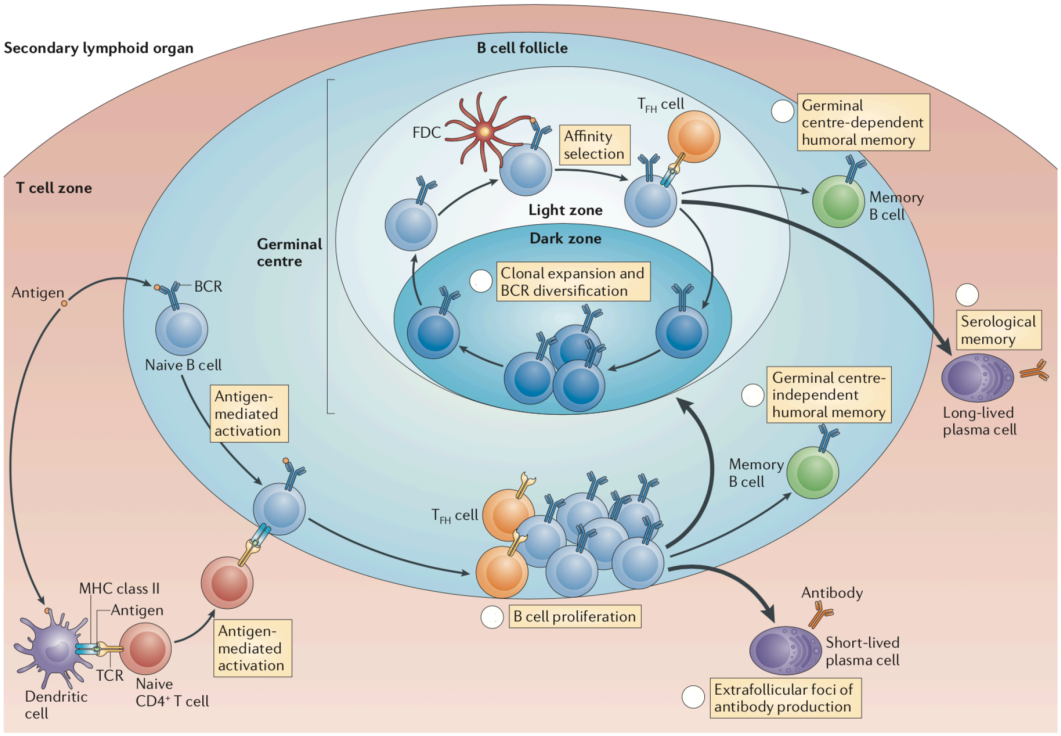

Figure 1.1 Representation of T-dependent (TD) immune response. B cells engulf antigen, process and present peptides by MHC II on cell surface and move to T-B cells borders. Previously activated T cells recognise peptides presented by MHC II and co-stimulate B cells by CD40-CD40L and cytokines. Fully activated B cells move to GC to undergo series of differentiation and proliferation (Kurosaki T. et al 2015, "Memory B cells", Nature Reviews, DOI: https://doi.org/10.1038/nri3802)

Second, is the T-independent response (TI) where antigens alone are able to initiate B cell activation exemplified in two types; TI-1 and TI-2. TI-1 responses are a consequence of binding of antigen to innate receptors on B cells and an example of this is LPS binding to TLR4 in mice. Clear examples of TI-1 responses are not apparent in humans. In human, capsular bacterial polysaccharides and polymeric flagella are considered TI-2 antigens. Their ability to initiate B cell activation is through their multivalent structure, which causes cross-linking of BCRs that activates B cells in the absence of cognate T cells help. Other cells such as dendritic cells and monocytes assist B cell response by expressing BAFF (B-cell activating factor), a ligand which binds to BAFF-R and TACI receptor, promoting B cell activation [3, 7]. T cells may also support the TI-2 response through the production of cytokines.

1.3 Marginal zone B cells

Marginal zone (MZ) cells are a B cell subset that reside the marginal zone surrounding the mantle zone in the white pulp of a spleen, and that circulate in the blood in humans [8]. Putative analogues have also been identified in the crypt epithelium of tonsil, the sub-capsular sinus in lymph node and in the dome area of Peyer's patches in gut associated lymphoid tissue of human intestine though how these relate to MZ B cells functionally is not clear [9, 10, 11]. MZ B cells' anatomical location ensures that they are the first B cells to encounter antigens [3]. Their functional importance relies on their ability to respond to TI-2 antigens. The marginal zone is fully formed within the first 2 years of life [12]. Thus, infants under two years old are more susceptible to infection with bacterial pathogens [13].

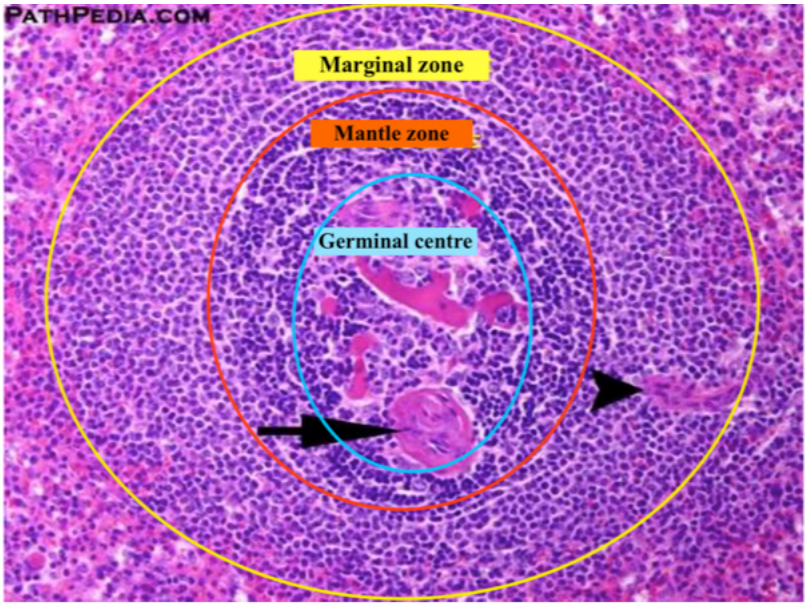

Figure 1.2 Staining of a spleen tissue section. The denser inner collection of tissue is the mantle zone while the paler outer collection of lymphoid tissue is the marginal zone. A germinal centre is formed around arterioles indicated by black arrow. https://www.pathpedia.com

1.4 Marginal Zone B cell subsets and development controversies

MZ B cells are morphologically larger in size than mantle zone B cells and phenotypically characterized as CD27+IgM+IgD+, while mantle zone B cells are CD27-IgM+IgD+ [7]. The significance of heterogenetic staining for surface IgD amongst CD27+IgM+ B cells reveals the presence of two B cell subsets that reside MZ; CD27+IgM+IgD+ and CD27+IgM+IgD- [10, 14]. Whilst there is general agreement that CD27+IgM+IgD+ are marginal zone B cells, whether the CD27+IgM+IgD- B cells are also in this group or if they represent memory B cells is

unclear and issues surrounding this are contentious [15]. Another highly controversial topic is whether MZ B cells have an independent developmental lineage in human. Around 95% of human MZ B cells have mutated Ig receptors and display a very low AID [16]. Also, CD27+IgM+IgD+ and CD27+IgM+IgD- subsets have mutations in the *Bcl6* gene [17]. These results favour the proposal of MZ B cells being germinal centre (GC) derived cells after the acquisition of somatic hyper-mutation. On the other hand, evidence shows that, human blood has CD27+IgM+IgD+ B cell subset that phenotypically corresponds to the splenic marginal zone B cells and are involved in T-independent immune response [18]. Moreover, Descatoire et al 2014 and Bemark 2015 identified a MZ precursor subset (CD27-CD45RB+) that, differentiates into MZ-like B cells by Notch2 ligation *in vitro*. This suggests that, MZ B cells have an independent development lineage [15, 19].

1.5 B cells surface molecules

The surface receptor TACI (Transmembrane activator and CAML interactor) is a tumour necrosis factor receptor homolog expressed by mature B cells including; marginal zone, memory B cells and plasma cells [20, 21, 22]. The interesting biology of TACI allows it to harbour two ligands BAFF and APRIL (**Figure 1.3**) [23, 24, 25]. TACI plays a critical role in the regulation of T-independent responses in the marginal zone B cells, which leads to survival, proliferation

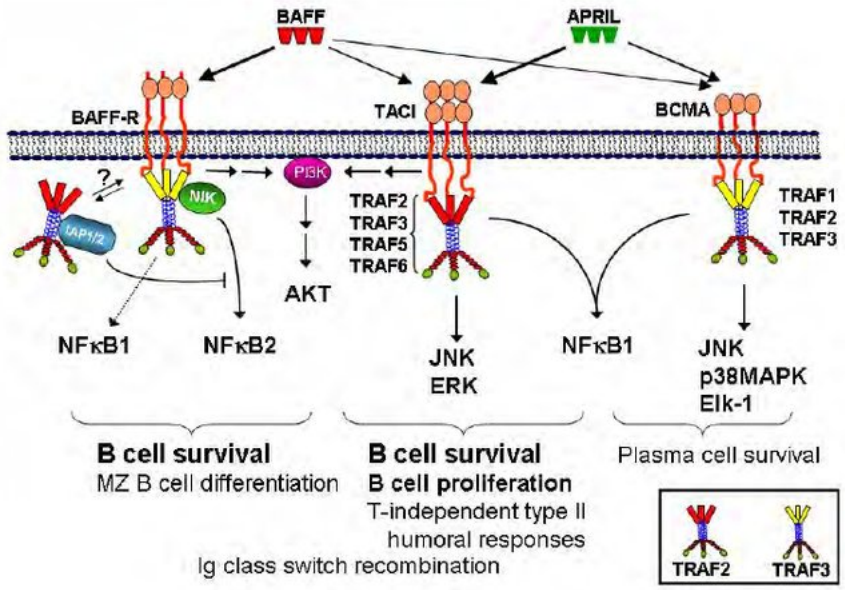

Figure 1.3 Schematic illustration of BAFF-R, TACI and BCMA receptors and their legends and signal transduction pathways. APRIL is a ligend for TACI and BCMA. BAFF is a ligend for TACI, BAFF-R and BCMA (Gema Pérez-Chacón and Juan M. Zapata 2012 "Mouse Models of Chronic lymphocytic leukemia", DOI: 10.5772/27091).

and isotype switching in B cells [7, 26, 27] via its ability to activate NF-kB and AP-1 transcription factors [28]. Furthermore, FcRL4 (Fc receptor-like protein 4) is another surface receptor expressed by marginal zone B cells in the intraepithelial and sub-epithelial regions of the mucosa-associated lymphois tissue (MALT) [29]. FcRL4 binds to aggregated IgA. It contributes to the homoeostasis environment of MALT by the inhibition of B cell signalling via its ITIM motifs [30].

1.6 Background on monoclonal antibody 4D12

In the 1980's our group generated a monoclonal antibody 4D12 (4D12 MoAb) by immunising BALB/c mice with low grade MALT lymphoma. By histology, 4D12 MoAb recognised an antigen on surface of cells residing the MZ in human spleen as well as a subset of GC cells selectively located in the light zone (**Figure 1.4**). They showed that, 4D12 MoAb had an effect on the mitogenic activity of *Staphylococcus aureus* Cowan I (SAC) by enhancing tonsil B cell activation. 4D12 MoAb was reported to recognise a protein 120-160kDa under non-reducing condition and 70-80kDa under reducing condition.

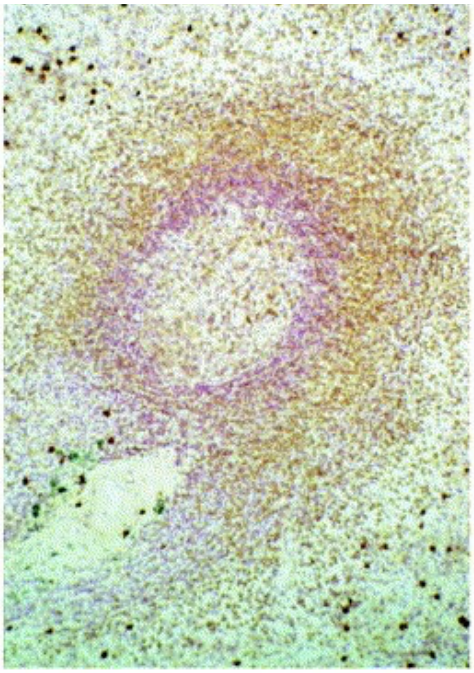

Figure 1.4 Immunoperoxidase-stained spleen tissue section with 4D12 MoAb. 4D12 MoAb bound to cells residing MZ and a subset of B cells in the GC, stated in brown (Spencer et al 1998 "Human marginal-zone B cells", Immunology Today, DOI:https://doi.org/10.1016/S0167-5699(98)01308-5).

1.7 Aims of the thesis

A B cell antigen selectively expressed by B cells in the splenic marginal zone and light zone of the GC has not been described since this antibody was produced and this antibody therefore remains unique and the antigen it recognises is uncharacterised. 4D12 MoAb has the potential to serve as a marginal zone lineage marker according to criteria that could resolve a number of current issues in the field and may have broader application if it is understood better.

The work in this thesis aims to:

1. Increase the understanding of the lymphocytes' expression of 4D12 by flow cytometry.
2. Investigate the nature of the 4D12 antigen by mass spectrometry and flow cytometry.

1.8 Hypothesis

4D12 is a monoclonal antibody that recognises a novel antigen expressed on the surface of B cells residing marginal zone and a subset of germinal centre cells.

Chapter 2:
Material and Methods

2.1 *Tissue and blood samples*

Tonsil samples were collected from surgical theatres at Guy's hospital. Samples were collected from healthy patients with sleep apnea. None were inflamed. Spleen samples were collected from cadaver organ donors. Peripheral blood mononuclear cell (PBMC) samples were from blood cones provided by National Health Service (NHS). All samples were used with Research Ethics Committee approval and patient's consent. All samples were treated anonymously by a given numerical code.

2.2 *High concentration monoclonal 4D12 antibody*

10mg FITC-conjugated, 10mg Biotin-conjugated and purified 4D12 monoclonal antibodies were generated and purchased from Davids Biotechnologie GmbH, Germany.

2.3 *ELISA assay*

ELISA plates were coated with 100 µl (1:5000) polyclonal goat anti-mouse immunoglobulins (Dako) in coating buffer pH 9 and incubated overnight in 2-4°C. Plates were washed x3 times with ELISA buffer 0.05% Tween20 (Sigma Aldrich) in 1x PBS (Life Technologies) pH 7.2. Non-specific binding was blocked with 10% skimmed milk in ELISA buffer for 1 hour at room temperature. The content was tipped out and plates were washed with ELISA buffer x3 times. 100 µl of hybridoma cells supernatant was added into 3 wells and incubated for 1 hour in room temperature. For positive control, 100 µl of (1:50) commercial monoclonal mouse anti-human MNF116 (Dako) antibody of known concentration was used to make a standard curve. For negative control RPMI medium was used. Content was tipped out and plates were washed with ELISA buffer x3 times. 100 µl of 1:5000 polyclonal rabbit anti-mouse immunoglobulins HRP (Dako) was added to each well and incubated for 1 hour at room temperature. Plates were washed with ELISA buffer x3 times. 100 µl of SIGMAFAST OPD substrate (Sigma) was added into each well and incubated for 20 mins in the dark at room temperature. Reaction was stopped by 50 µl H_2SO_4. Optical density was measured by Benchmark Plus microplate spectrophotometer (Bio-Rad) at 492nm.

2.4 *Tissue section staining*

Frozen sections (6 μm) of normal tonsil were cut onto glass slides by the Cancer Centre Biobank facility and stored at -80^{o}C. Immediately before use, sections were thawed and circled with a wax pen and then fixed in fresh acetone for 30 minutes at room temperature. Sections were then equilibrated in 1X PBS for 5 minutes before the addition of 100 μl of 4D12 supernatant for 1 hour. Sections were then washed x3 with PBS before addition of 100 μl of rabbit anti-mouse immunoglobulin conjugated to peroxidase. The distribution of 4D12 was visualized (brown) with a Dako substrate kit and sections were counterstained with haematoxylin before mounting.

2.5 *Surface staining (flow cytometry)*

Freshly thawed aliquots of spleen, tonsil and blood cells were transferred into 5 ml polystyrene tube (Falcon), spun down at 4^{o}C 400g for 7 min and stained with CD19-PerCP/Cy5.5, CD27-APC, IgD-APC/Cy7, IgM-BV711, CD24-PECy7, CD21-PE, TACI-PE, FcRL4-PECy7, CD10-PECy7 (Biolegend), CD38-PE (eBioscience) and 4D12-FITC (**Table 2.1**) in FACS staining buffer with 2% FBS and 1mM EDTA for 15 min at 4°C in the dark. Samples were washed with 2 ml FACS buffer, spun down for 7 min and re-suspended in 400μl of 1μg/ml DAPI (Biolegend) for LIVE/DEAD stain before analysed by FACS CANTO II machine (BD Bioscience). Appropriate isotype controls and fluorescence minus one controls (FMO) were used for all samples. Spleen, tonsil and blood were kept on ice for the whole the protocol. Data was analysed using FlowJo 10.3 program.

2.6 *B cell subsets gating strategy*

Figure 2.2 illustrates the gating technique used to identify B cell subsets. Gating starts with lymphocyte, single cells, live cell and B cells; CD19+ (**Figure 2.1**). Marginal zone (MZ); CD27+IgM+IgD-/+, class switched memory (CS); CD27+IgM-IgD-, plasmablast; CD27++CD38++, mature naïve; CD27-IgM+IgD+ and transitional B cells (T1); CD27-CD38++CD24++, (T2); CD27-CD38+CD24+. 4D12 expression from each B cell subset was measured using isotype control.

2.7 *Intracellular staining (flow cytometry)*

Freshly thawed aliquots of spleen, tonsil and blood cells were washed in 1X PBS, spun down at 4ºC 400g for 7 min and resuspended in (1:200) Zombie Aqua in PBS for 30 min at 4°C in the dark for LIVE/DEAD stain. Samples were washed with 2 ml FACS buffer with 2% FBS and 1mM EDTA, spun down for 7 min and stained with surface antibodies CD19-PerCP/Cy5.5, CD27- APC, IgD-APC/Cy7, IgM-BV711, CD10-PECy7 (**Table 2.2**) in FACS staining buffer for 15 min at 4°C in the dark. Samples were washed with 2 ml FACS buffer, spun down for 7 min and re-suspended in 2% paraformaldehyde (Alfa Aesar) in PBS for 30 min 4°C in the dark. Samples were washed with PBS, spun down at 4ºC 400g for 7 min and resuspended in 1X permeabilisation buffer (invitrogen) in PBS for 30 min at 4°C in the dark. Samples were washed with PBS, spun down at 4ºC 400g for 7 min and stained with 4D12-FITC (**Table 2.2**) in permeabilisation buffer for 30 min at 4°C in the dark. Samples were then washed with 2 ml FACS buffer, spun down for 7 mins and re-suspended in 400µl FACS buffer and analysed by FACS CANTO II machine (BD Bioscience). Appropriate isotype controls and FMO controls were used for all samples. Spleen, tonsil and blood were kept on ice for the whole the protocol. Data was analysed using FlowJo 10.3 program.

Figure 2.1

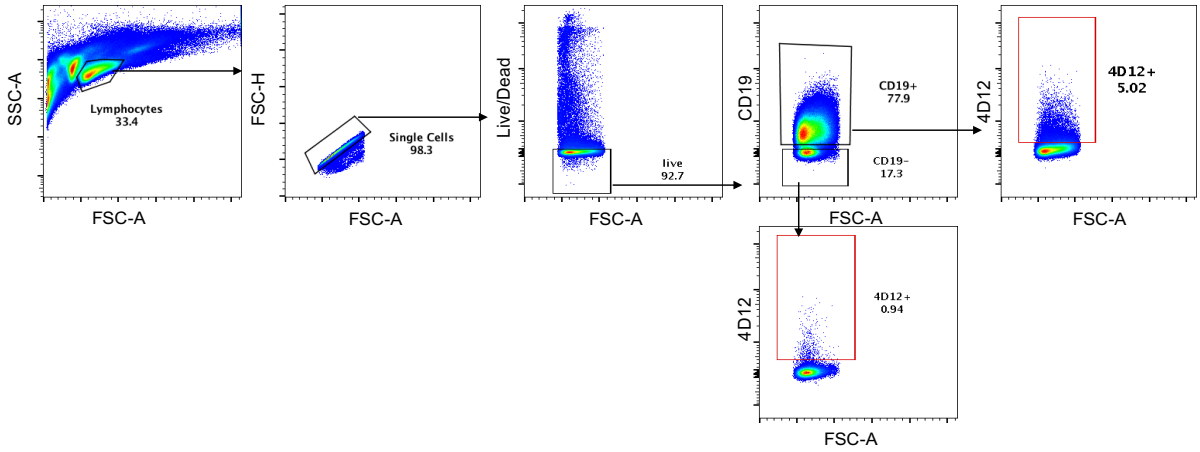

Figure 2.1 Gating strategy of CD19+ and CD19- expression of 4D12 in spleen. The lymphocyte gate was identified by visualisation of SSC-A and FCS-A. Single cells were selected by visualising FSC-H and FSC-A. Live cells were those that excluded the DAPI stained cells. CD19+ and CD19- cells were then identified and used for further B cell subset. IgG2b-FITC isotype control was used to gate on 4D12+ population indicated in red.

Figure 2.2

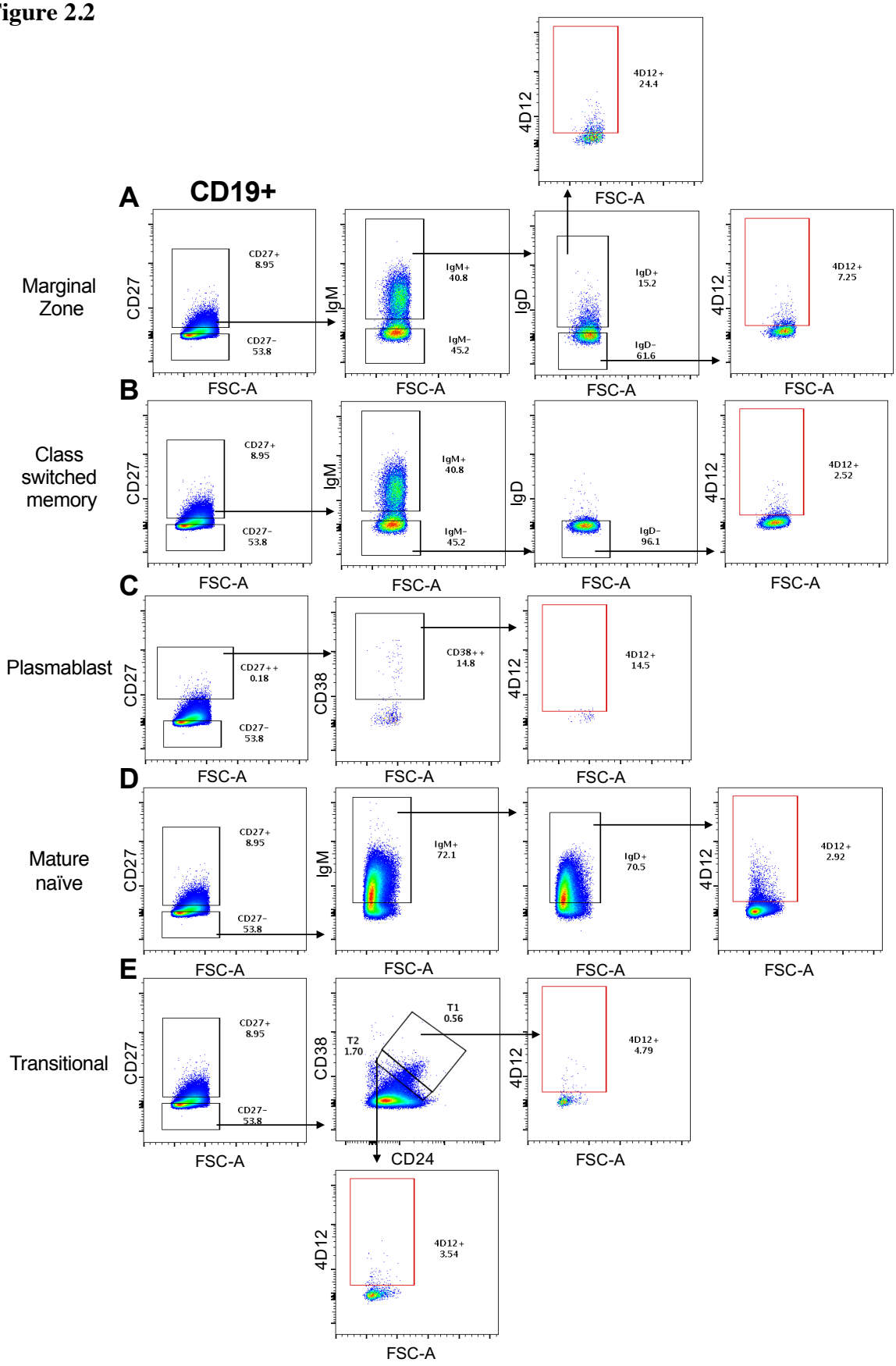
A
CD19+
Marginal Zone
CD27
FSC-A
CD27+ 8.95
CD27- 53.8
IgM
IgM+ 40.8
IgM- 45.2
IgD
IgD+ 15.2
IgD- 61.6
4D12
4D12+ 24.4
4D12+ 7.25
B
Class switched memory
CD27+ 8.95
CD27- 53.8
IgM+ 40.8
IgM- 45.2
IgD- 96.1
4D12+ 2.52
C
Plasmablast
CD27++ 0.18
CD27- 53.8
CD38
CD38++ 14.8
4D12+ 14.5
D
Mature naïve
CD27+ 8.95
CD27- 53.8
IgM+ 72.1
IgD+ 70.5
4D12+ 2.92
E
Transitional
CD27+ 8.95
CD27- 53.8
CD38
T1 0.56
T2 1.70
CD24
4D12+ 4.79
4D12+ 3.54

Figure 2.2. Gating strategy for different B cell subsets of CD19+ cells in spleen pre- gated as in Figure 3.3 (**A**) marginal zone; CD27+IgM+IgD+ and CD27+IgM+IgD- (**B**) class switched memory; CD27+IgM- IgD- (C) plasmablasts; CD27++CD38++ (**D**) mature naïve; CD27-IgM+IgD+, (**E**) transitional B cells (T1); CD27- CD24++CD38++, (T2); CD27-CD24+CD38+. Gates highlighted in red are 4D12+ cells of each B cell subset.

Table 2.1 markers used to identify B cells subsets and surface 4D12 antigen.

Panel 1	Panel 2	Panel 3
CD19 - PerCP/Cy5.5 (Biolegend)	CD19 - PerCP/Cy5.5 (Biolegend)	CD19 - PerCP/Cy5.5 (Biolegend)
CD27 – APC (Biolegend)	CD27 – APC (Biolegend)	CD27 – APC (Biolegend)
IgD - APC/Cy7 (Biolegend)	IgD - APC/Cy7 (Biolegend)	IgD - APC/Cy7 (Biolegend)
IgM - BV711 (Biolegend)	IgM - BV711 (Biolegend)	IgM - BV711 (Biolegend)
CD38 – PE (eBioscience)	CD21 – PE (Biolegend)	TACI - PE (Biolegend)
CD24 - PECy7 (Biolegend)	FcRL4- PECy7 (Biolegend)	CD10- PECy7 (Biolegend)
4D12 – FITC (Davids Biotech.)	4D12 - FITC (Davids Biotech.)	4D12 – FITC (Davids Biotech.)

Table 2.2 markers used to identify B cells subsets and intracellular 4D12 antigen.

Panel 4
CD19 - PerCP/Cy5.5 (Surface) (Biolegend)
CD27 – APC (Surface) (Biolegend)
IgD - APC/Cy7 (Surface) (Biolegend)
IgM - BV711 (Surface) (Biolegend)
CD10 - PECy7 (Surface) (Biolegend)
Zombie Aqua (Surface) (Biolegend)
4D12 – FITC (Intracellular) (Davids Biotech.)

Chapter 3:

Re-establishment of 4D12 MoAb secreting clone and testing antibody binding activity

3.1 Introduction

Monoclonal antibody 4D12 was reported in 1990 to recognise a surface antigen on a subset of human B cells by flow cytometry and B cells in the marginal zone of the human spleen and a subset of germinal centre B cells by immunohistochemistry [31]. It was shown to co-stimulate B cells in culture with SAC and therefore appeared to be potentially a functionally important antibody. At the time that this antibody was described, many functionally important immunological receptors had not been described. For example, Toll-Like receptors (TLR) were not described until 1996 [32]. SAC is a complex T-independent B cell mitogen. It can activate B cells by cross-linking cell surface immunoglobulins via Protein A [33]. SAC can also activate B cells by sensitising B cells to recognise cell wall-associated lipopeptides using TLR2, which subsequently leads to B cell proliferation [34, 35]. Therefore, the previously reported SAC co-stimulation experiment with 4D12 MoAb may have represented co-stimulation between the 4D12 antigen and a TLR and/or surface immunoglobulin. The molecular weight was estimated to be approximately 120-160kD under non-reducing condition and around 80kD under reducing condition, but the antigen remained unidentified.

The aim of the work in this chapter was to bring the 4D12 cell line from storage and to characterise its reactivity by flow cytometry according to a fuller set of parameters than was available when the hybridoma was first made. Amongst the parameters investigated were TACI and FcRL4. TACI was included due to its role in the regulation of MZ B cell T-independent responses by its interaction with TLR's such as TLR-2 via mTOR resulting in proliferation and class switching of MZ B cells [22, 36]. In addition, FcRL4 was investigated because it is associated with intraepithelial and sub-epithelial MZ B cells in MALT and its potential role in B cell signalling inhibition via ITIM when binding its ligand, aggregated IgA [29, 30].

3.2 Results

3.2.1 *Re-establishment of the 4D12 clone following long term storage*

A vial of cells labelled 're-cloned' and dated 1997 were taken from storage in liquid N_2. Peritoneal exudate cells (PEC) from one mouse were divided between 2 x 96 U shaped well plates and cells from the thawed vial were divided between the wells of one of the 96 well plates. After 2 weeks, cells from some of the wells containing 4D12 hybridoma and PEC showed growth of cells visible as pellets to the naked eye and as clusters of cells under the microscope. These cells were divided between the wells of the second 96 well plate so that they remained viable whilst the supernatants were collected and tested for antibody secretion.

3.2.2 *Hybridoma cells supernatant tests showed the secretion of antibody with previously described properties of 4D12 MoAb*

Secretion of antibody by hybridoma cells was investigated using an ELISA assay developed for this project. Commercial monoclonal mouse antibody of known concentration was used to make the standard curve, and RPMI medium was used for the negative control. The optical density reading of supernatants tested showed the presence of mouse Ig's in the supernatant of the wells containing growing cells at 0.8 μg/ml (**Figure 3.1A**). To examine if the monoclonal antibody bound B cells as previously described, a tissue section of tonsil sample was stained using the 4D12 hybridoma cells supernatant and inspected under light microscope. **Figure 3.1B** displays a clear 4D12 MoAb staining on some of GC cells in brown consistent with the earlier observations. These results confirmed the production of 4D12 MoAb by the hybridoma cells and its binding to a subset of germinal centre cells as previously described.

3.2.3 *Clone expansion to generate high concentrations of specifically purified antibody*

Since the clone, on revisiting, still appeared to have interesting properties and there are current controversies in B cell biology that this reagent could be applied to, clones were sent to a biotechnology company to be grown up to generate sufficient antibody for affinity purification and labelling. A FITC-conjugated, biotin-conjugated and purified mouse 4D12 MoAbs at high concentration were received 2 months after.

3.2.4 *Titration of 4D12 MoAb*

To titrate the optimal binding concentration of 4D12 MoAb with the least non-specific binding, frozen samples of viable tonsil cells were thawed out, washed and stained with the FITC-conjugated 4D12 MoAb at 20 μg/ml, 10 μg/ml, 5 μg/ml, 2.5 μg/ml and 1.25 μg/ml. A mouse IgG2b isotype control conjugated to FITC was used at the same concentrations. The flow cytometric data indicated the best binding activity of 4D12 MoAb was at 10 μg/ml (**Figure 3.2**). This concentration was then used to stain all samples.

3.2.5 *4D12 MoAb recognised surface antigen on B cells*

To test if 4D12 MoAb is B cell specific by flow cytometry, frozen aliquots of spleen, tonsil and blood cells were thawed out, washed then stained with CD19 antibody and 4D12 MoAb. The 4D12 MoAb predominantly recognised a surface antigen on CD19+ rather than CD19- cells (**Figure 3.3**) in spleen, tonsil and blood. This suggested that, 4D12 MoAb recognised an antigen mainly expressed on the surface of B cells.

3.2.6 *Identification of B cell subsets in spleen, tonsil and blood*

Before the analysis of the percentage of B cell subsets expressing the 4D12 antigen, the relative average sizes of B cell subsets in each tissue studied were considered. **Figure 3.4A** shows that, the mature naïve subset was the largest B cell subset in spleen, tonsil and blood. The subsets CD27+IgM+IgD+, CD27+IgM+IgD- and class switched (CS) memory B cells tended to be more abundant in spleen than in blood. Transitional B cells (T1) and (T2) were proportionally higher in blood compared to the spleen or tonsil. GC cells were greater in frequency in spleen and tonsil than in blood. Finally, plasmablasts were present with low frequency in spleen, tonsil and blood.

3.2.7 *4D12 MoAb distinguished surface antigen on MZ cells and different B cell subsets*

To investigate the expression of the 4D12 antigen by different B cell subsets by flow cytometry, cryopreserved spleen cells (n=7), tonsil cells (n=6) and PBMC's (n=6) were stained with 4D12 MoAb and a panel of antibodies to discriminate between different B cell subsets (**Table 2.1**). the flow cytometric data showed that, 4D12 MoAb recognised a proportion of CD27+IgM+IgD+ and CD27+IgM+IgD- cells in spleen, tonsil and blood (**Figure 3.4B**). Moreover, an average of approximately 13% of GC B cells in spleen was recognised by 4D12 MoAb though a lower percentage of cells with GC phenotype expressed the 4D12 antigen in

blood and tonsil. The percentage of plasmablasts in spleen and tonsil recognised by 4D12 MoAb was high, though the actual number of 4D12 antigen+ (4D12+) plasmablasts was likely to be low due to their small population size (**Figure 3.4A**). In contrast, the percentage of plasmablasts recognised by 4D12 MoAb in blood was low. T1 and T2 B cell expression of the 4D12 antigen was observed in spleen, tonsil and blood (**Figure 3.4B**). CS memory and mature naïve B cells in all samples showed relatively low percentages of 4D12+ cells (**Figure 3.4B**). The mean florescence intensity (MFI) of 4D12 antigen expression on the surface of all B cell subsets showed similar expression level of antigen in spleen and blood (**Figure 3.4C**). This was not the case in tonsil, where T1 B cells showed a higher level of 4D12 antigen expression when compared to the other tonsillar B cells subsets. Next the average frequencies of B cells with the phenotypes: CD27+IgM+IgD+, CD27+IgM+IgD-, mature naïve and CS memory B cells in the total 4D12+ and CD19+ populations were visualised as pie charts. **Figure 3.4E** illustrates that, although the CD27+IgM+IgD+ and CD27+IgM+IgD- populations had the greatest increase in frequencies of 4D12+ cells, over half of the 4D12+ cells did not have these phenotypes because the populations were relatively less abundant (**Figure 3.4A**).

3.2.8 *Comparison of surface 4D12 antigen expression by B cells in spleen, tonsil and blood*

The expression of the surface 4D12 antigen by CD27+IgM+IgD+, CD27+IgM+IgD- and mature naïve was compared between spleen, tonsil and blood. As shown in **Figure 3.5A** there was a consistent tendency for more CD27+IgM+IgD+ and CD27+IgM+IgD- cells to be recognised by 4D12 MoAb in spleen than in tonsil and blood. This was statistically significant for the CD27+IgM+IgD- population (P= 0.014) in tonsil. Moreover, there was no statistical difference in the expression level of 4D12 antigen by CD27+IgM+IgD+, CD27+IgM+IgD- and mature naïve cells (**Figure 3.5B**) in spleen, tonsil and blood. This data suggested that the expression of 4D12 antigen might be associated with tissue location of B cells.

3.2.9 *4D12+ cells expressed TACI and FcRL4*

The relationship between 4D12 antigen expression and surface molecules such as TACI and FcRL4 that are expressed by B cells in the MZ was investigated. Cryopreserved spleen cells (n=7), tonsil cells (n=6) and PBMC's (n=6) were thawed and stained for the 4D12 antigen, B cell subsets markers, TACI and FcRL4 (**Table 2.1**). **Figure 3.6A** shows a greater frequency of TACI expressing cells in the 4D12+ compared to the 4D12- population and mature naïve B cells in spleen. 4D12+ cells in tonsils also showed TACI expression, however it was a smaller proportion than in spleen. CD27+IgM+IgD+ and CD27+IgM+IgD- populations had similar

proportions of TACI expressing cells compared to the 4D12+ population in spleen, but this was less evident in tonsil samples. As reported elsewhere [37], blood cells in general tended to include a low frequency of TACI expressing cells compared to tissues. Moreover, significantly more 4D12+ cells expressed FcRL4 when compared to 4D12- cells in all tissues (**Figure 3.6B**). FcRL4 expressing cells were most abundant in tonsils that have an epithelial area. Overall these results indicated that 4D12+ cells were more phenotypically similar to CD27+IgM+IgD+ and CD27+IgM+IgD- in including the highest frequencies of cells expressing TACI and FcRL4 compared to mature naïve.

3.2.10 *Intracellular 4D12 MoAb staining*

Intracellular staining was carried out in order to examine the possibility of intracytoplasmic antigen expression by B cells. Frozen tonsil sample was thawed out, washed and divided into two groups. First group was stained with CD19 antibody and 10 μg/ml 4D12 MoAb for surface expression. Second group was stained for surface CD19 antibody, then cells were fixed, permeabilised and stained with 4D12 MoAb at the same concentration for intracellular expression. Surprisingly, 4D12 MoAb bound to an intracellular antigen in all CD19+ and CD19- cells (**Figure 3.7B**), this contrasted with 10.3% and 1.65% of CD19+ and CD19- cells respectively, being 4D12+ at same concentration on the cell surface (**Figure 3.7A**). This suggested that 4D12 might be an intracellular antigen and more widely distributed internally than previously thought.

3.2.11 *Intracellular titration of 4D12 MoAb*

To optimise binding concentration of 4D12 MoAb with the least non-specific binding, frozen tonsil samples were thawed out, fixed, permeabilised and stained intracellularly with the FITC-conjugated 4D12 MoAb at 1.25 μg/ml, 0.6 μg/ml, 0.15 μg/ml, 0.075 μg/ml and 0.037 μg/ml. For control, a mouse IgG2b isotype conjugated to FITC at the same concentrations was used. The flow cytometric data indicated the best intracellular binding activity of 4D12 MoAb was 0.15 μg/ml (**Figure 3.8**). This concentration was then used to stain all samples intracellularly for further investigation.

3.2.12 *4D12 MoAb intracellular antigen recognition*

Since 4D12 MoAb recognised an antigen that appeared to be proportionally more in the cytoplasm, the recognition of 4D12 MoAb to the intracellular antigen by CD19+ and CD19- population was sought by flow cytometry. Cryopreserved spleen cells (*n*=3), tonsil cells (*n*=3) and PBMC's (*n*=3) were thawed out, fixed, permeabilised and stained with 4D12 MoAb intracellularly and a panel of surface antibodies to discriminate between different B cell subsets (**Table 2.2**). A relatively high frequency of both CD19+ and CD19- cells in spleen bound to the 4D12 MoAb compared to surface stain. 4D12 MoAb recognition to a cytoplasmic antigen was lower in tonsil where statistically more CD19+ cells expressed 4D12 antigen than CD19- cells. A lower proportion of peripheral CD19+ cells tended to be 4D12+ in comparison to CD19- (**Figure 3.9A**). The MFI of 4D12 antigen expression showed no difference in the expression level of the antigen between all samples (**Figure 3.9B**). Subsequently, the recognition of 4D12 MoAb to the intracellular antigen by the different B cell subsets was examined. **Figure 3.9C** shows that 4D12 MoAb recognised a relatively great proportion of all splenic B cell subsets. On the other hand, the percentages of 4D12+ cells within B cell subsets in the blood was variable. 4D12 MoAb recognised an intracellular antigen in all blood B cell subsets, however, it recognised a very high percentage (approx. 95%) of subsets such as mature naïve and CS memory or very low proportion of GC and plasmablasts at individual events. Moreover, 4D12 MoAb recognised a greater proportion of tonsillar GC and plasmablasts when compared to mature naïve. It also, recognised a percentage of CD27+IgM+IgD+, CD27+IgM+IgD-, CS memory and transitional B cells (**Figure 3.9C**). The overall trend of MFI indicated that 4D12 antigen was expressed in a similar manner within all B cell subsets in spleen, tonsil and blood (**Figure 3.9D**). These data suggested the intracellular expression of 4D12 antigen by all B cell subsets and non-B cells in spleen, tonsil and blood.

3.2.13 *Comparison of intracellular 4D12 antigen expression by B cells in spleen, tonsil and blood*

The intracellular expression of 4D12 antigen by B cells subsets (including: CD27+IgM+IgD+, CD27+IgM+IgD-, mature naïve, CS memory, GC, and transitional B cells) was compared in spleen, tonsil and blood. The flow cytometric results showed a constant trend toward the recognition of 4D12 MoAb to a greater proportion of all splenic B cell subsets than tonsil and blood counterparts. This is statistically shown in **Figure 3.10**.

3.3 Discussion

Monoclonal antibody 4D12 was re-established and the cell reactivity of the antibody characterized by flow cytometry. A surprising observation in this chapter was the intracellular staining of 4D12 MoAb by both B and T cells. This contrasted with the surface staining observed that was restricted to B cells and that recognised some B cell subsets more than others consistent with the published paper [31]. The use of relatively low concentration 0.15 μg/ml to optimally detect the intracellular antigen expression in comparison to 10 μg/ml for optimal surface staining indicated the weak distribution of 4D12 antigen on the surface of some B cells and its rich availability in the cytoplasm.

The intracytoplasmic staining of 4D12 antigen in most lymphocytes observed here is inconsistent with the observations made in tissue sections in the literature and in the histology based antibody screening test reported here. Although the staining in tissue sections could be intracellular, it does not appear to have a broad distribution by B cells and T cells in tissue sections but is rather restricted to the germinal centre and marginal zone. It is possible that the antigen has a broad distribution but, for example, the antigen may have been destroyed by fixation of frozen sections before staining. Fixation with organic solvents such as; acetone might have a slight effect on the morphology of the section by either cross-linking or denaturing of proteins. This could be explored by using different fixatives though it was not possible to explore this possibility in the project. It is also possible that the intracellular staining by 4D12 MoAb was an artefact caused by fixation and or permeabilisation of the cells, though the experiment was well controlled by an isotype control antibody and it is not clear how this could happen.

The previous literature described the binding of 4D12 MoAb to MZ and a subset of GC cells but not cells in mantle zone or T zone in spleen [31]. If only the surface staining is considered, the current data supported that, since 4D12 MoAb mainly recognised a surface antigen on CD19+ (a marker found on the surface of B cells) cells, but not CD19- cells (**Figure 3.3**). Moreover, **Figure 3.4B** showed the most surface recognition of 4D12 MoAb to a proportion of CD27+IgM+IgD+, CD27+IgM+IgD- in spleen, tonsil and blood as well as a lower proportion of GC, mature naïve and CS memory. This data is consistent with the previously published literature on the presence of some CS memory and mature naïve B cells in the MZ [38 39]. The fact that, 4D12 MoAb was generated by immunising mice with a low-grade MALT

lymphoma, which is a malignancy of B cells of the marginal zone in the mucosa- associated lymphoid tissue [40] supports the idea that, 4D12 could be an antigen expressed on the surface of marginal zone B cells.

A greater frequency of surface 4D12+ cells expressed TACI in spleen in contrast to 4D12- cells and mature naïve (**Figure 3.6A**), but less proportionally in tonsil. Moreover, relatively high proportion of 4D12+ cells expressed FcRL4 (**Figure 3.6B**) in tonsils, which has an epithelial region, whereas a lower frequency of 4D12+ cells expressed FcRL4 in spleen and blood. Proportionally similar to 4D12+ cells, CD27+IgM+IgD+ and CD27+IgM+IgD- cells expressed TACI and FcRL4. These results support the concept that 4D12+ cells are phenotypically similar to CD27+IgM+IgD+ and CD27+IgM+IgD- by their surface expression.

The surface staining showed that, 4D12 MoAb recognised a notable percentage of GC cells in spleen but a very low percentage of GC in tonsil. However, the tissue section staining of tonsil samples showed binding of 4D12 MoAb to GC cells (**Figure 3.1B**). It is unlikely for two different assays to show a contrary outcome, nonetheless, the 4D12 antigen might be better expressed on the surface of GC cells in spleen than in tonsil. It is also possible that GC may differ in different anatomical contexts in terms of the number of 4D12+ cells they contain. Furthermore, the frequency of plasmablasts recognised by 4D12 MoAb in tissue (spleen and tonsil) was high, however, plasmablasts occupy a small proportion of the total B cell population in tissue (**Figure 3.4A**), a low percentage of plasmablasts were 4D12+ in blood. Likewise, transitional B cells were proportionally small in population size, yet a great frequency was 4D12+. Therefore, the surface and intracellular expression of 4D12 by plasmablasts and transitional cells was proportionally low.

In conclusion, the re-establishment of 4D12 clone after a long-term storage has shown the ability of hybridoma cells to secret 4D12 MoAb that recognised cells in the MZ and some of GC cells by immunohistochemistry. Moreover, flow cytometric data showed that, 4D12 MoAb recognised an antigen on surface of B cell. Of the total B cells, surface staining showed that, 4D12 MoAb recognised a greater proportion of CD27+IgM+IgD+, CD27+IgM+IgD- and transitional cells in spleen, tonsil and blood. It also, recognised a great frequency of plasmablasts in spleen and tonsil, but very low frequency in blood and very low proportion of CS memory and mature naïve B cells. On the other hand, the intracellular staining for 4D12 antigen showed abundancy of antigen expression in the cytoplasm by all B cell subsets and the

T cells in spleen and a lower percentage in tonsil and blood. Furthermore, there was a tendency of higher percentages of splenic CD27+IgM+IgD+ and CD27+IgM+IgD- cell to be 4D12+ in contrast to tonsil and blood analogues by both surface and intracellular staining. A notable proportion of 4D12+ cells expressed TACI and FcRL4 as compared to 4D12- cells, which make them phenotypically similar to CD27+IgM+IgD+ and CD27+IgM+IgD- cells by their surface expression.

Figures (Chapter 3)

Figure 3.1

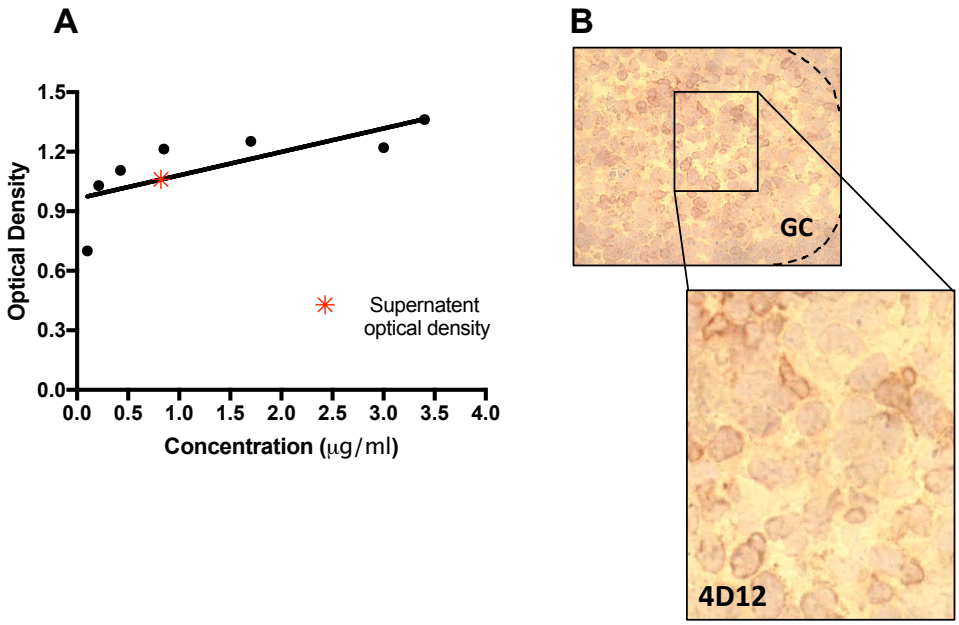

Figure 3.1. Secreted 4D12 MoAb recognised target antigen on cell surface of B cells **(A)** ELISA assay analysis of selected healthy clone's supernatant. Standard curve of a known commercial monoclonal mouse antibody and supernatant from wells containing hybridoma cell growth **(B)** Microscopic images (original magnification x200) of immunoperoxidase-stained tonsil tissue section. 4D12 MoAb (hybridoma supernatant) bound to B cell subset in the GC, stated in brown.

Figure 3.2

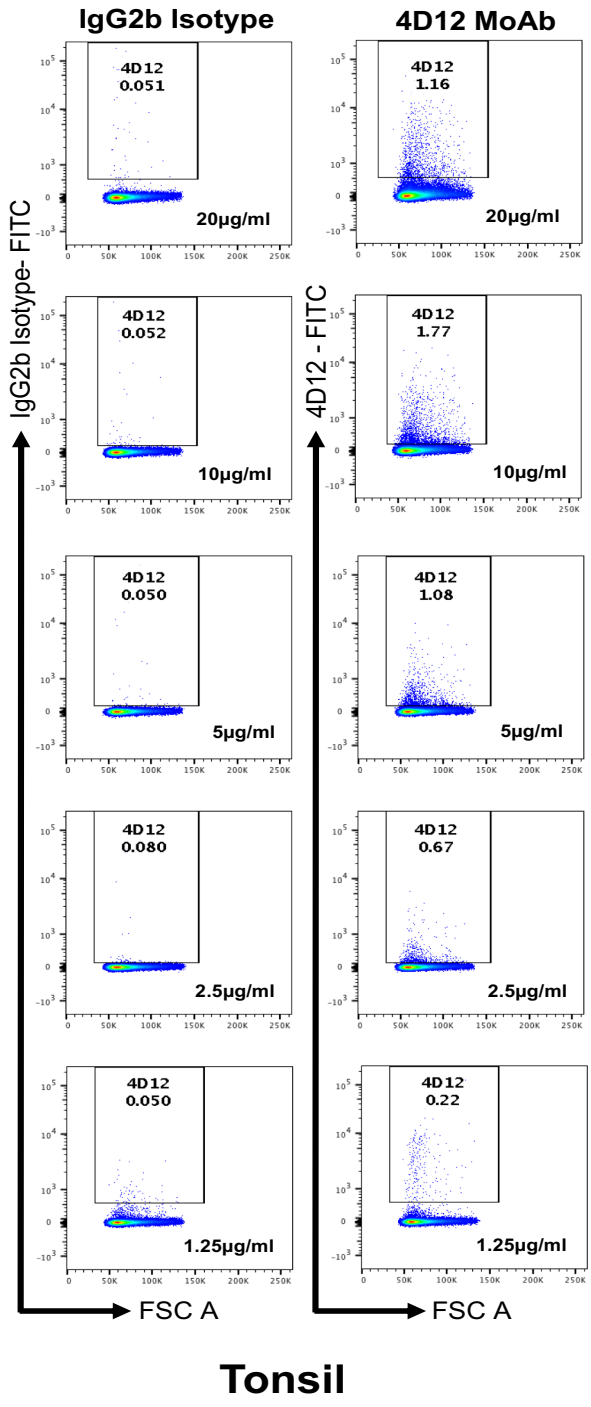

Figure 3.2. Titration of 4D12 MoAb by flow cytometry. Tonsil cells were stained with 4D12 MoAb-FITC at 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1.25 µg/ml. For control IgG2b-FITC isotype was used at same concentrations. The highest percentage of 4D12 MoAb binding when the isotype control at the same concentration detected 0.03% of the total population was 10 µg/ml.

Figure 3.3

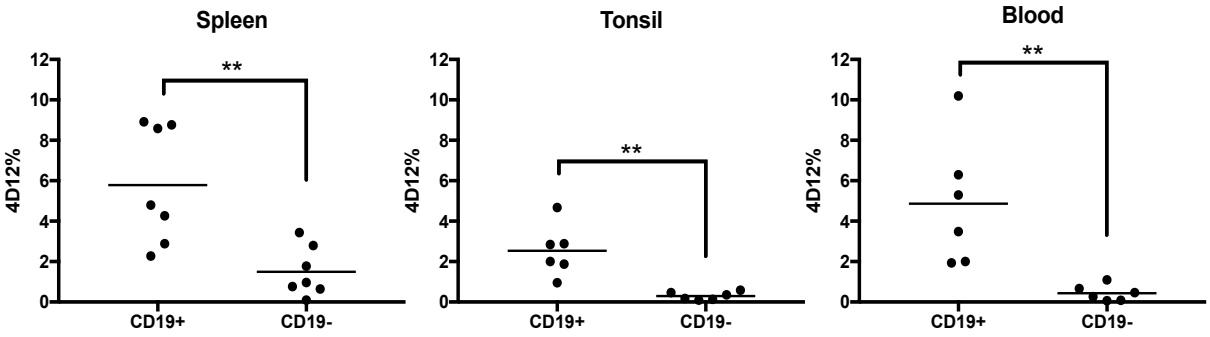

Figure 3.3. Analysis of the proportion of CD19+ (B cells) and CD19- (T cells) expressing the 4D12 antigen. 4D12 MoAb recognised a greater proportion of CD19+ cells than CD19- cells in spleen (*P*= 0.0036), tonsil (*P*= 0.0016) and blood (*P*= 0.0065). Spleen (*n*= 7) tonsil (*n*=6) PBMC's (*n*=6). Paired T test was used for statistical analysis.

Figure 3.4

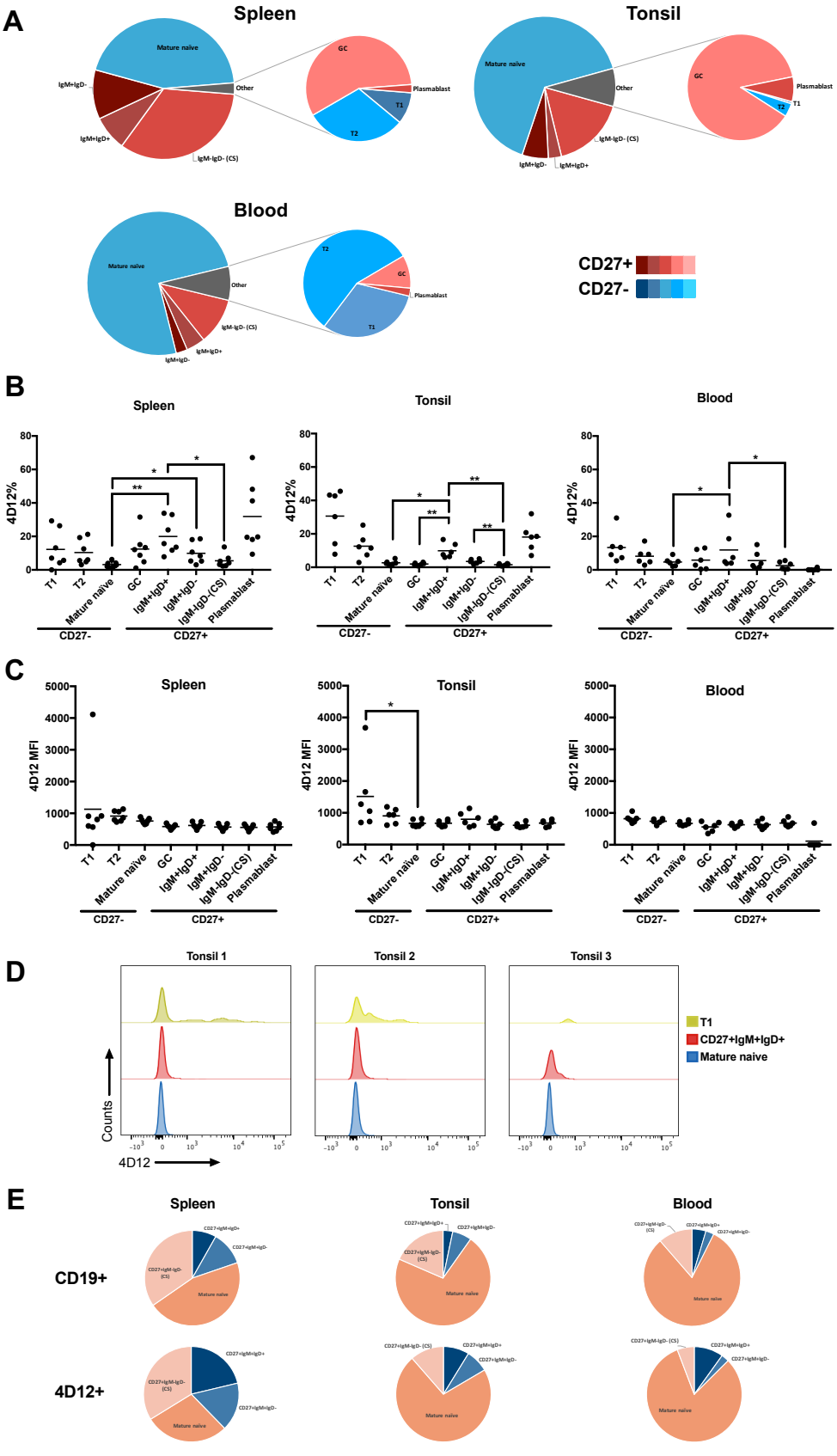
A
Spleen
Tonsil
Blood
Mature naive
Other
GC
Plasmablast
T1
T2
IgM+IgD-
IgM+IgD+
IgM-IgD- (CS)
CD27+
CD27-
B
Spleen
Tonsil
Blood
4D12%
T1
T2
Mature naive
GC
IgM+IgD+
IgM+IgD-
IgM-IgD-(CS)
Plasmablast
CD27-
CD27+
C
Spleen
Tonsil
Blood
4D12 MFI
D
Tonsil 1
Tonsil 2
Tonsil 3
T1
CD27+IgM+IgD+
Mature naive
Counts
4D12
E
Spleen
Tonsil
Blood
CD19+
4D12+

Figure 3.4. Proportion and visualization of relative numbers of B cells expressing the 4D12 antigen. (**A**) Pie charts depicting the proportion of different B cell subsets in spleen, tonsil and blood. (**B**) 4D12 MoAb recognised CD27+IgM+IgD+ and CD27+IgM+IgD- cells in spleen, tonsil blood. GC B cells are recognised by 4D12 MoAb in spleen and blood. Low frequency of GC B cells was recognised by 4D12 MoAb in tonsil. Plasmablasts in spleen and tonsil were 4D12+. Very low proportion or none of plasmablasts in blood were 4D12+. Transitional B cells (T1) and (T2) were recognised by 4D12 in spleen, tonsil and blood. Mature naïve and class switched memory B cells showed very low recognition by 4D12 MoAb in all samples. (**C**) No difference in 4D12 expression level between any of the B cell subsets in spleen and blood. Higher 4D12 antigen expression level by T1 B cells in tonsil than other tonsillar B cell subsets. (**D**) MFI histogram charts of 3 tonsil samples showing the different of 4D12 antigen expression between T1, CD27+IgM+IgD+ and mature naïve B cells. (**E**) A higher CD27+IgM+IgD+ and CD27+IgM+IgD- proportions recognised by 4D12 MoAb compared to CD19 antibody in spleen, tonsil and blood. Majority of 4D12+ cells belong to mature naïve and CS memory B cells combined. Spleen (n= 7) tonsil (n=6) PBMC's (n=6). For statistical significance, T-test was used for statistical significance in all samples (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

Figure 3.5

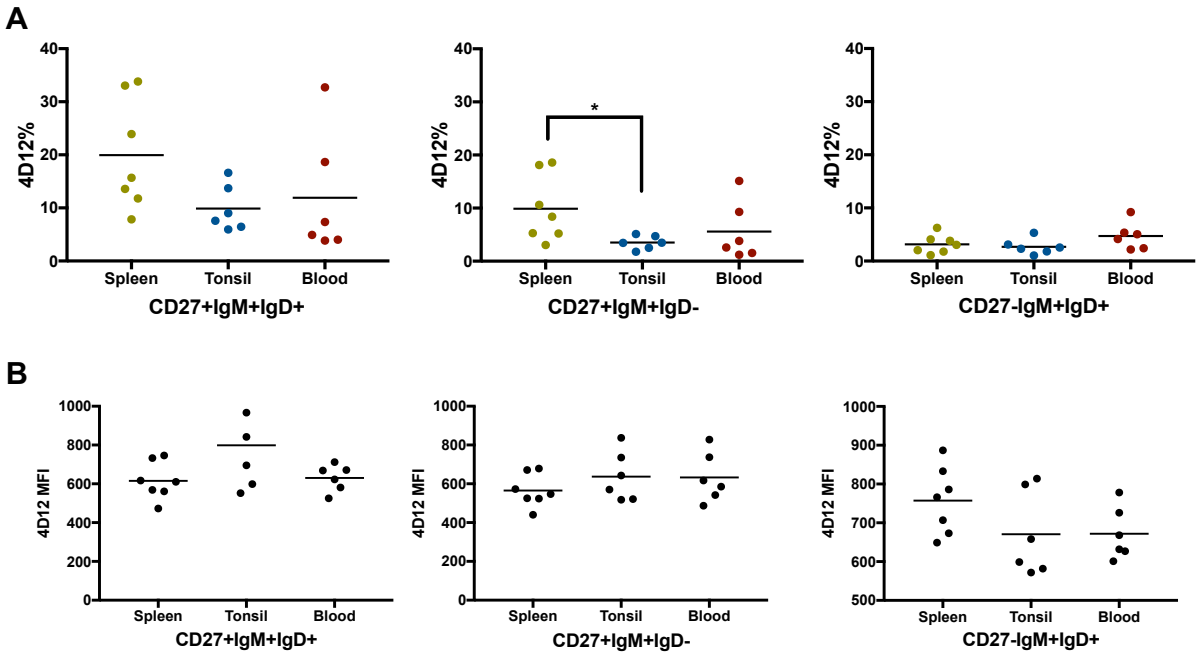

Figure 3.5. Comparison of frequencies of cells expressing 4D12 antigen in CD27+IgM+IgD- , CD27+IgM+IgD- and CD27-IgM+IgD+ (mature naïve) B cells in spleen, tonsil and blood by flow cytometry. (**A**) 4D12 MoAb recognised higher percentages of CD27+IgM+IgD+ and CD27+IgM+IgD- cells in spleen, a lower percentage in tonsil and blood. Very low proportions of mature naïve were 4D12+ in spleen, tonsil and blood (**B**) The MFI showed no difference in expression level of 4D12 antigen by CD27+IgM+IgD-, CD27+IgM+IgD- and mature naïve in spleen, tonsil and blood. Spleen (*n*= 7) tonsil (*n*=6) PBMC's (*n*=6). Unpaired T-test was used for statistical significance (* $P \leq 0.05$)

Figure 3.6

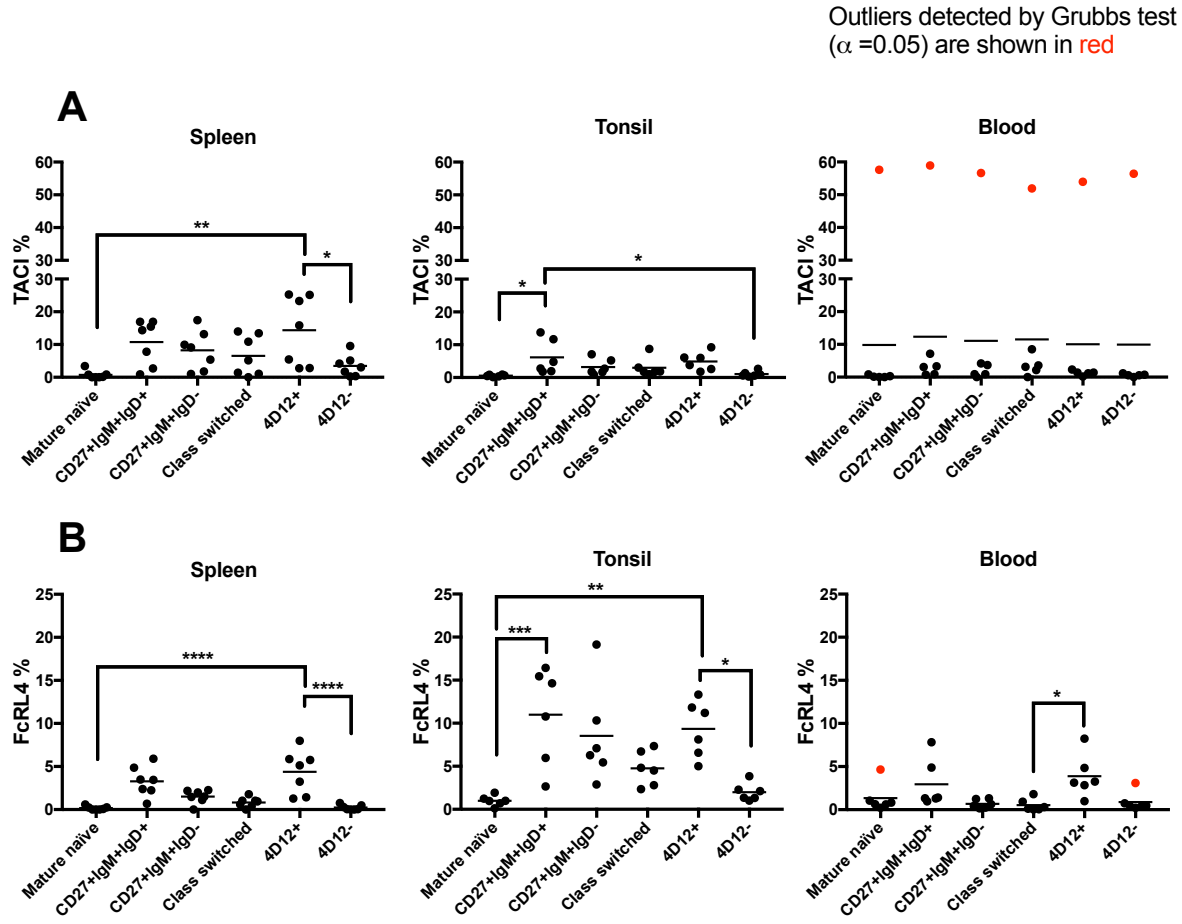

Figure 3.6. Analysis of the proportion of 4D12+ cells expressing TACI and FcRL4. (**A**) 4D12+ cells expressed greater frequencies of TACI in spleen and tonsil in contrast to 4D12- cells. Low TACI expression in 4D12+ cells in blood. CD27+IgM+IgD+, CD27+IgM+IgD- and CS memory expressed TACI in spleen and lower percentage in tonsil and blood. Very low TACI expression by mature naïve B cells. (**B**) 4D12+ cells expressed FcRL4 in spleen, blood and more abundant proportion in tonsil. Great frequency of CD27+IgM+IgD+, CD27+IgM+IgD- and CS memory expressed FcRL4 in tonsil and a lower proportion in spleen and blood. Spleen (n= 7) tonsil (n=6) PBMC's (n=6). For statistical significance T-test was used for statistical significance in all samples (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

Figure 3.7

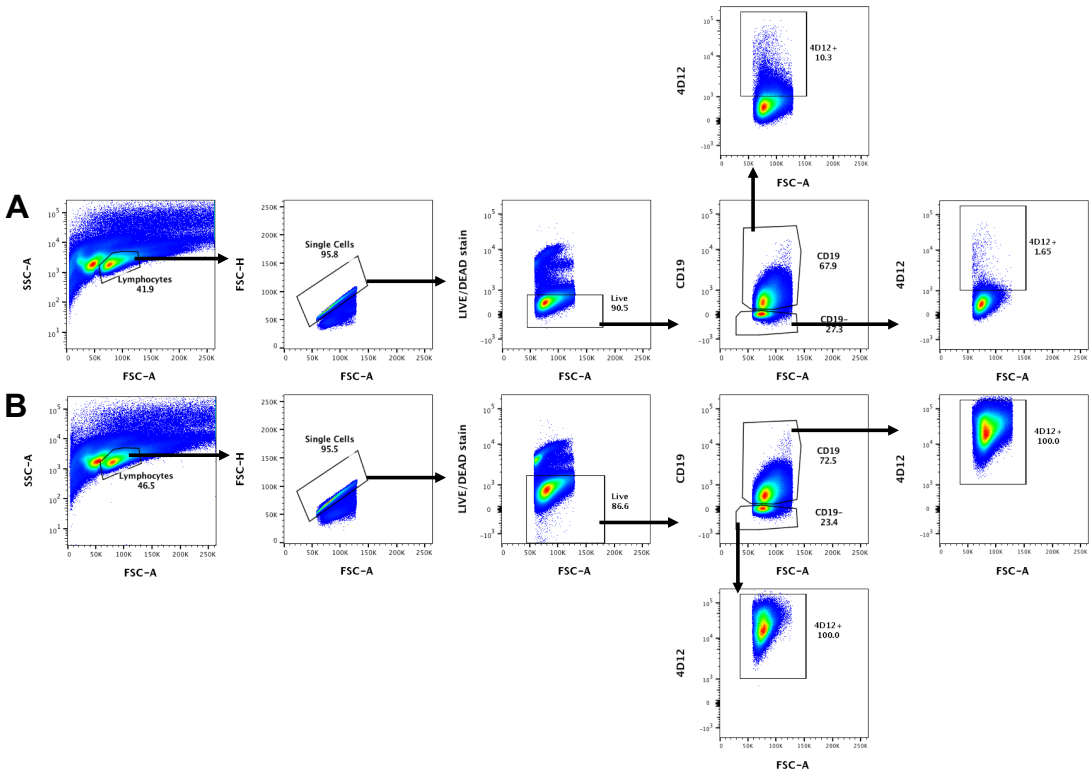

Figure 3.7. Frequencies of CD19+ and CD19- populations expressing 4D12 antigen by surface and intracellular staining using the same antibody concentration 10 µg/ml. (**A**) Surface staining of 4D12 MoAb: 10.3% of CD19+ and 1.65% of CD19- populations were 4D12+. (**B**) Intracellular staining of 4D12 MoAb: both CD19+ and CD19- were 100% 4D12+.

Figure 3.8

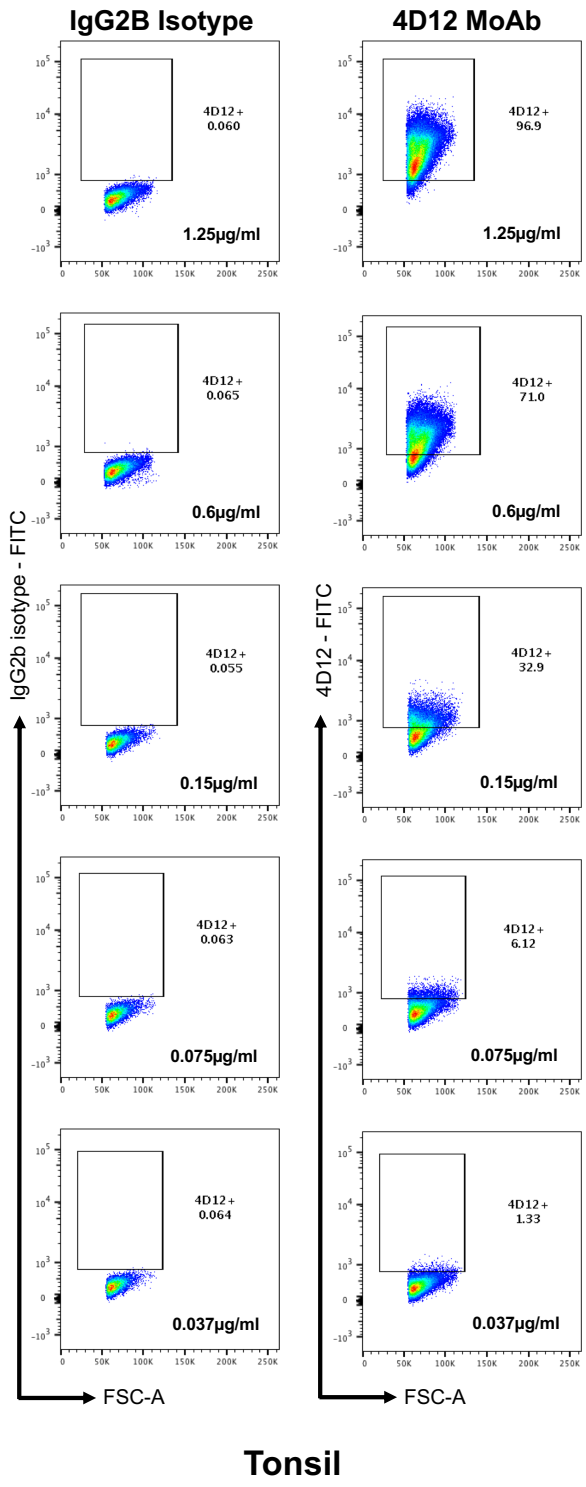

Figure 3.8. Titration of intracellular 4D12 MoAb by flow cytometry. Tonsil cells were thawed out, fixed, permeabilised and stained with 4D12 MoAb-FITC at 1.25 µg/ml, 0.6 µg/ml, 0.15 µg/ml, 0.07 µg/ml and 0.037 µg/ml. For control IgG2b-FITC isotype was used at same concentrations. The best 4D12 MoAb binding activity when the isotype control at the same concentration detected 0.06% of the total population was 0.15 µg/ml.

Figure 3.9

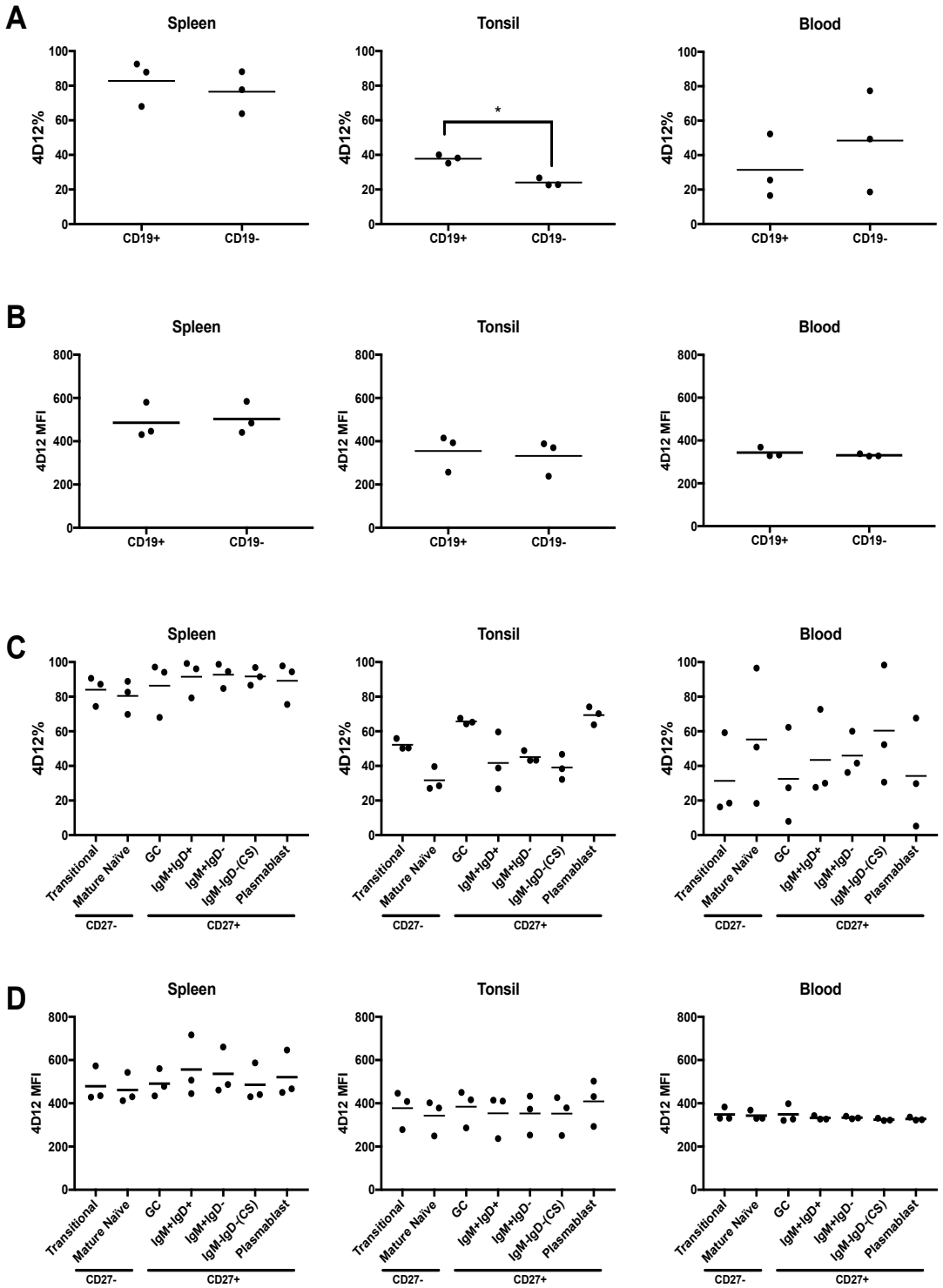
A
Spleen
Tonsil
Blood
4D12%
CD19+
CD19-
*
B
4D12 MFI
C
Transitional
Mature Naïve
GC
IgM+IgD+
IgM+IgD-
IgM-IgD-(CS)
Plasmablast
CD27-
CD27+
D

Figure 3.9. Intracellular visualization of relative percentages of CD19+, CD19- and B cell subsets expressing the 4D12 antigen. (**A**) Intracellularly, 4D12 MoAb recognised similar frequencies of CD19+ and CD19- population in spleen. 4D12 MoAb recognised a higher proportion of CD19+ than CD19- in tonsil, while it recognised greater proportion of CD19- than CD19+ cells in blood. (**B**) No difference in 4D12 antigen expression level between CD19+ and CD19- in spleen, tonsil and blood. (**C**) 4D12 MoAb recognised a very high proportion of all splenic B cell subsets. 4D12 MoAb recognised great proportions of tonsillar plasmablasts and GC. It also recognised a percentage of CD27+IgM+IgD+, CD27+IgM+IgD- , CS memory and transitional B cells and a lower percentage of mature naïve in tonsil. 4D12 MoAb recognised different frequencies of cells within each B cells in each blood sample. (**D**) No significant difference in 4D12 antigen expression level between any B cell subsets in spleen, tonsil and blood. Spleen (n= 3) tonsil (n=3) PBMC's (n=3). T-test was used for statistical significance in all samples (* $P \leq 0.05$).

Figure 3.10

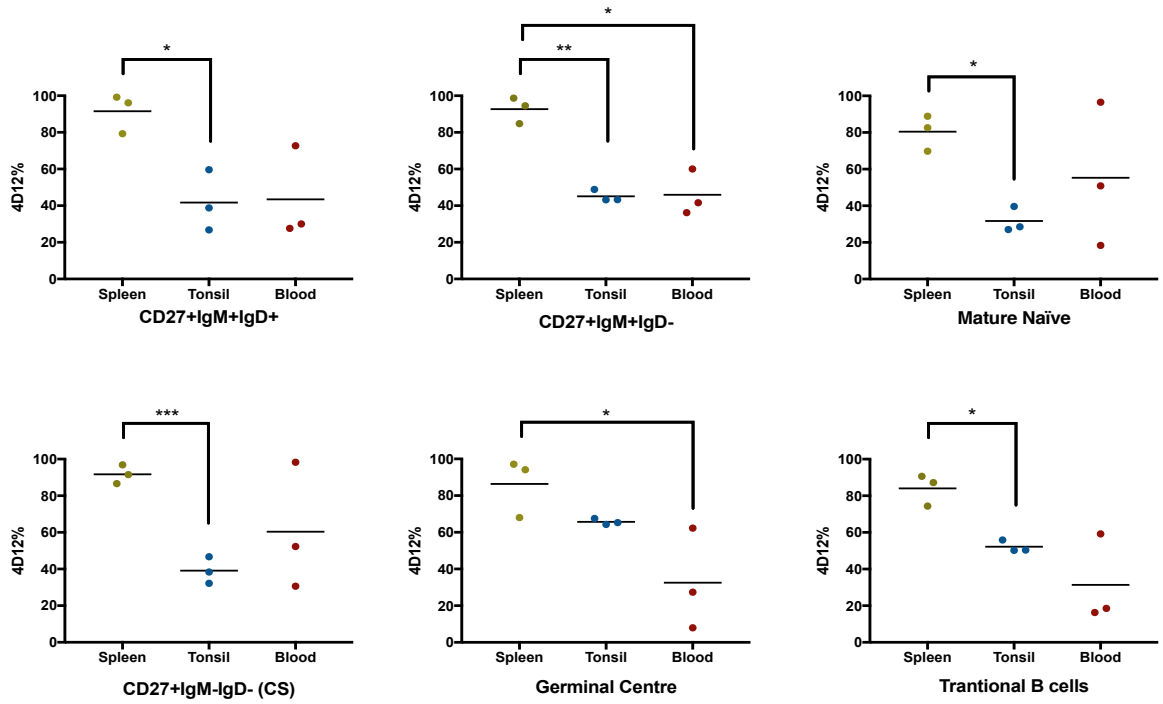

Figure 3.10. Comparison of cell proportions expressing intracellular 4D12 antigen in B cell subsets including (CD27+IgM+IgD- and CD27+IgM+IgD-, mature naïve, CS memory, GC and transitional) among spleen, tonsil and blood by flow cytometry. 4D12 MoAb recognised higher frequencies of all splenic B cells subsets when compared to tonsil and blood in all B cell subsets. Spleen (n= 3) tonsil (n=3) PBMC's (n=3). Unpaired T-test was used for statistical significance in all samples (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

Chapter 4:

Analysis of the mass spectrometry outcomes and testing for 4D12 antigen identity

4.1 Introduction

In chapter 3, the reactivity of 4D12 MoAb was investigated and shown to recognise an antigen on surface of B cells and in the cytoplasm of all lymphocytes. However, the identity of the 4D12 antigen is still unknown.

To initiate characterisation of the antigen, aliquots of 4D12 MoAb were shared with a collaborator who tested the reactivity of 4D12 MoAb via western blot. Reactivity with a lysate of healthy tonsil cells, tonsil from patient with Chronic lymphocytic leukaemia (CLL), and YT NK-like cell line were tested. The expression of 4D12 antigen was detected as a single clean band in the healthy tonsil sample (**Figure 4.1A**) and a very thick band by the CLL sample (**Figure 4.1B**) at approximately 35kDa, but very low or negative expression from control (YT NK-like cell). Previous literature reported that 4D12 MoAb recognised proteins of 120 – 160 kDa under non-reducing condition and 70 - 80kDa under reducing condition [31]. This was done by immunoprecipitation of antigen, using 4D12 MoAb coupled to Sepharose beads. Protein targets were eluted, and immunoprecipitated proteins were removed from the beads by boiling. Samples were separated on a gradient gel. Since the western blot data were clear and consistent we decided to use this for the next stage of analysis.

Next, the lysate of CLL and YT NK-like cell line were separated on 1D SDS-PAGE gel and two areas one above 35kDa and one below 35kDa were cut from the gel (**Figure 4.1D**) and given to a second collaborator at Centre of Excellence for Mass Spectrometry for further characterisation. A file was provided as the output of the mass spectrometry which was the starting point of the analysis in this chapter. The aim of this chapter is to analyse and test the outcomes of mass spectrometry by flow cytometry. The possibility that two antibodies recognised by flow cytometry depends on the fact that they will bind to antigen proportionally resulting in a diagonal line on the flow plot. This method is relatively cheap, simple and effective way to test for identity of an antigen.

4.2 Results

4.2.1 *Analysis of mass-spectrometry data*

Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis successfully identified many proteins from the gel bands after searching against the Uniprot Human database. Database generated files were uploaded into Scaffold 4 (v4.8.4) software. Qualitative protein assignments from sequence data following LC-MS/MS analysis produced a total of 256 possible protein assignments from CLL sample subsequent to database searching against Uniprot Human at 95% CI probability and a total of 303 possible protein assignments from YT NK-like cell line at the same stringency.

A plan was made to investigate the mass spectrometry analysis. The Scaffold 4 data for protein candidates to CLL sample were analysed manually based on 4 parameters. First, peptides coverage; which is the number of amino acids in all found peptides of a specific protein divided by the total number of amino acids in the entire protein sequence. The higher the coverage the better sequence alignment. All protein assignments were screened for amino acid sequence alignment with CLL sample provided (**Figure 4.2**). A minimum of 4% peptides coverage was considered as the lowest limit of coverage for a potential target.

Second, mainly unique protein assignments to the CLL sample were considered, however, shared assignments between CLL and the control with a high peptide coverage such as Glyceraldehyde-3-phosphate dehydrogenase (85% coverage) were considered (**Figure 4.3**). Unique protein assignments to control only were excluded.

Third, molecular weight; candidates with a same/similar molecular weight to 35kDa were selected. Two gel bands were submitted for mass spectrometry (above 35kDa and below 35kDa). Therefore, any candidate's molecular weight that were similar in size to 35kDa were selected. Candidates that had high peptide coverage but were larger than 39 kDa or smaller than 31kDa were excluded (**Table 4.1**). This information was obtained from the Genecard website.

Fourth, plausibility of the candidate location; the presence of a candidate in both cytoplasm and cell surface was essential. This was considered because previous chapter showed the

expression of 4D12 antigen in cytoplasm and cell surface. Potential candidates were narrowed down to 13 (**Table 4.1**).

4.2.2 *Testing of unverified antibodies by flow cytometry*

Unconjugated antibodies (Rabbit polyclonal and Mouse IgG1 monoclonal antibodies) against the 13 selected possible candidates were purchased from ProteinTech Europe. Since 4D12 MoAb is IgG2b, binding of 4D12 MoAb would not interfere with binding of secondary antibody. Not all antibodies were specifically validated for flow cytometry. To examine antibody reactivity by flow cytometry, a tonsil sample was thawed out, cells were fixed, permeabilised and stained with 13 different unconjugated antibodies at the recommended manufacturer concentration. Cells were washed, and binding was detected with either PE anti-rabbit antibodies or APC anti-mouse IgG1 antibodies. Two control types were used; PE anti-rabbit antibody was used to detect purified rabbit polyclonal isotype control and FMO control. **Figure 4.4** shows that only Immunity associated protein 1 (GIMAP1) antibody did not exhibited any reactivity to its antigen because 0.069% of lymphocytes were positive (marked in red). This is because GIMAP1 expression was mainly reported to be in spleen and lymph node [41]. No current data show expression of GIMAP1 in tonsil. This test showed that 12 out of 13 antibodies detected antigen by flow cytometry.

4.2.3 *Intracellular examination for 4D12 antigen by flow cytometry*

After the testing of the 13 antibodies, selected candidates were tested by flow cytometry to identify if they could share the identical target with 4D12 MoAb. In this case, a diagonal line of the flow cytometry plot would be expected that resemble the illustration in **Figure 4.5**. The recognition of two antibodies (for example; any antibody of the 12 and 4D12 MoAb) to the same target provides a diagonal line based on the equal proportional expression.

To examine that tonsil and blood cells were thawed, fixed, permeabilised and stained for each individual target candidate together with 4D12 antigen. Flow cytometric data showed a very similar expression of Cathepsin Z (CTSZ) to 4D12 antigen in tonsil creating a diagonal line (**Figure 4.7F**). However, the blood sample did not have the same result (**Figure 4.6F**). In addition, there was a tendency toward the same expression of Cytosolic Fe-S cluster assembly factor (NUBP1) and 4D12 antigen in both tonsil (**Figure 4.7L**) and blood (**Figure 4.6L**). Other

expression profiles indicated that there was no similarity to 4D12 antigen. These results suggested 1 candidate that could be the antigen recognised by 4D12 MoAb. Further investigation is required.

4.3 Discussion

Mass spectrometry identified many potential antigenic targets for 4D12 antigen, that were approximately 35kDa. A simple flow cytometric assay was used to seek the identity of the 4D12 antigen because this method was rapid and relatively inexpensive. However, it failed to conclusively identify a target for 4D12 MoAb. Nevertheless, the flow cytometric result suggested Cytosolic Fe-S cluster assembly factor (NUBP1) to be a possible target for 4D12 MoAb.

NUBP1 alongside NUBP2 constitute a heterotetrameric protein complex (P-loop NTPase) which is a component of the cytosolic iron-sulfur (Fe-S) protein assembly machinery [42]. It plays a role in the maturation of the extra-mitochondrial Fe-S clusters in mammalian cells. NUBP1 expression was mainly reported to be in the cytosol. Moreover, the depletion of NUBP1 resulted in an impairment of cell growth [43]. Fe-S clusters are ancient ubiquitous co-factors. They exert multiple functions in humans [44] including; gene regulation, sensors for oxygen and iron and they act as a catalytic centres [45]. In addition they have critical role in electron transfer reactions during oxidative phosphorylation process [46].

According to Genecards website, NUBP1 is ubiquitously expressed almost everywhere including B and T lymphocytes and is highly expressed in the lymph node. The results from the previous chapter showed an intracellular expression of 4D12 antigen in all lymphocytes in spleen, blood and tonsil samples which is consistent with the NUBP1 expression, however, other mammalian samples from different organs should be tested for 4D12 antigen expression. Furthermore, to accurately investigate the possibility of 4D12 antigen being NUBP1, western blotting could be used. The interaction between antibody and antigen could be tested by running a human commercial recombinant NUBP1 protein on SDS-PAGE gel. The recombinant NUBP1 protein could then be transferred on a western blot membrane. The membrane could be incubated with 4D12 MoAb. Binding of 4D12 MoAb to recombinant NUBP1 could be detected by conjugated secondary antibody.

If adequate funds and time were available, alternative methods could have been used to accurately investigate the identity of 4D12 antigen. A more sensitive way could be to immunoprecipitate the 4D12 antigen then submit protein targets for mass spectrometry analysis to find out the identity of 4D12 antigen. The advantage of the immunoprecipitation method

over western blot is to accurately target and elute 4D12 antigen, because many cellular proteins would have the same molecular weight of approximately 35kDa and using immunoprecipitation restricts the end product to be the targeted protein. The previous publication describing the 4D12 antigen that used immunoprecipitation suggested that it was bigger than the 35kDa that was explained here. More recent studies from collaborator found that 4D12 MoAb that is an IgG2b antibody did not work well for immunoprecipitation but worked better for western blotting. However, spending more time to optimise this could have had a better outcome and resolve this issue.

As reported in the literature, 4D12 MoAb has a co-stimulatory property on B cells when incubated with SAC [31]. Therefore, finding out the identity of 4D12 antigen is essential to examine the its functional importance. To investigate the functions of 4D12 antigen, many methods could be applied, such as the CRISPR system. If the 4D12 antigen coding sequence is known, guided Cas9 nuclease can be used to cut the known sequence, which will result in a knockout gene of the target antigen. Mutated cells can then be cultured in media with 4D12 MoAb or alone and outcomes can be investigated by flow cytometry assay.

Immunity associated protein 1 (GIMAP1) was eliminated from the selected candidates tested for 4D12 antigenic identity because the GIMAP1 antibody did not show any binding activity to antigen in tonsil (**Figure 4.3**), yet it could potentially still be a candidate. GIMAP1 is a member of GIMAP family of GTPases that is expressed in all lymphocytes [47]. It plays an important role in the growth and survival of T and B lymphocytes as well as formation of GC in mice [48, 49]. As stated earlier, GIMAP1 is mainly expressed in spleen and lymph node [41] and the previous chapter of this project showed a relatively higher proportion of lymphocytes that expressed 4D12 antigen in spleen in contrast to tonsil and blood. Therefore, it is important to investigate the identity of 4D12 antigen in spleen.

In conclusion, mass spectrometry produced many potential antigenic targets for 4D12 MoAb, that were approximately 35kDa. A simple flow cytometry assay could not confirm the identity of the 4D12 antigen. Nevertheless, it suggested a possible antigenic target for 4D12 MoAb to be NUBP1. If sufficient funds and time were provided an alternative accurate method could be used, such as immunopecipotation of 4D12 antigen, then finding out the identity of 4D12 antigen by mass spectrometry.

Figures (Chapter 4)

Figure 4.1

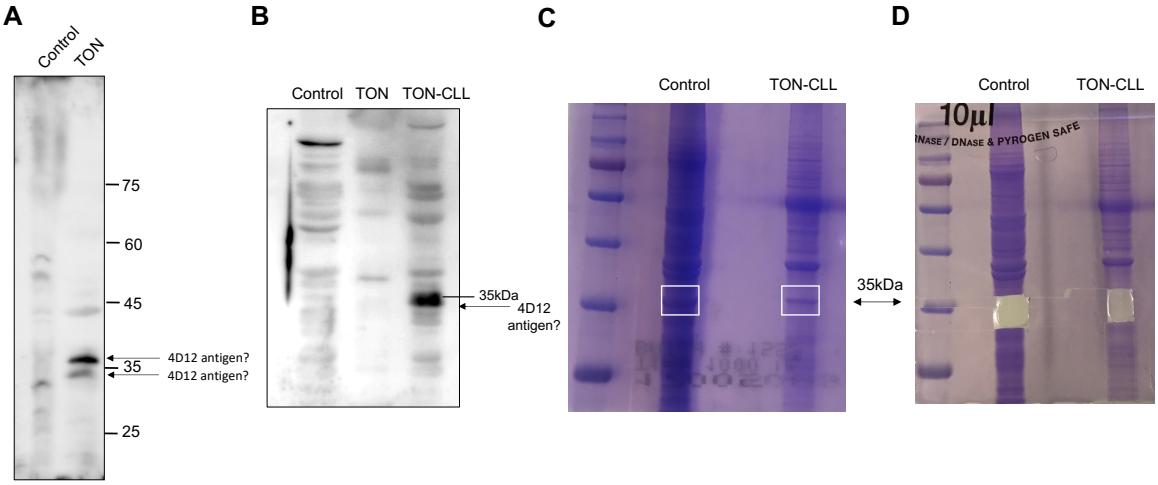

Figure 4.1. Imagination of a possible identity of the antigen recognised by 4D12 MoAb by western blot. (**A**) YT NK-like cell control and heathy tonsil, 90 µg lysate per lane. (**B**) YT NK-like cell control, healthy tonsil and CLL tonsil, 30 µg lysate per lane. The single strong band is around 35kDa identified by 4D12 MoAb in healthy and CLL tonsil samples. (**C**) YT NK-like cell control (60 µg per well) and CLL tonsil (30 µg per well) lysate on a 1D SDS PAGE gel, stained with Imperial Blue stain. (**D**) 4 pieces of gel were cut; one above and one below 35kDa for the control and one above and one below for the CLL tonsil.

Figure 4.2

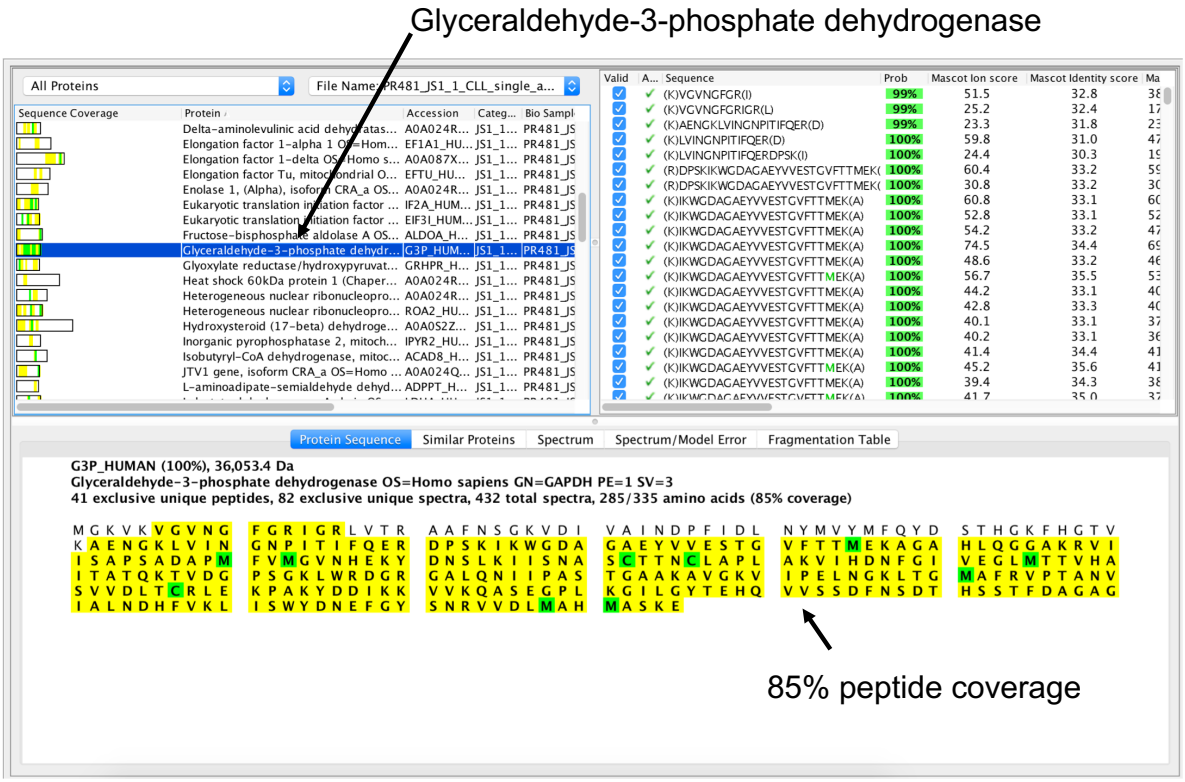

Figure 4.2. Demonstration of Scaffold 4 data analysis. Protein assignments from CLL sample were screened for peptide coverage. A minimum of 4% peptides coverage was considered as the lowest limit of coverage for a potential target.

Figure 4.3

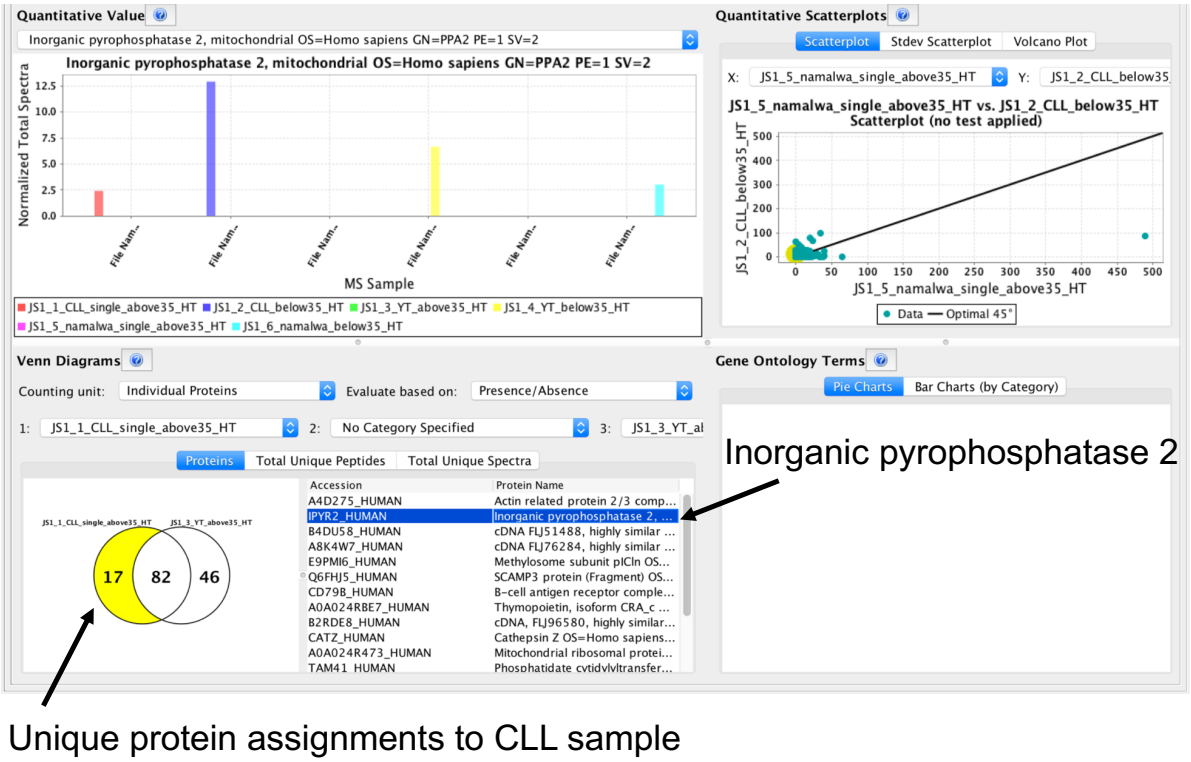

Figure 4.3. Demonstration of Scaffold 4 data analysis. Consideration of unique protein assignments to CLL sample (labeled in yellow), for example; Inorganic pyrophosphatase 2. Exclusive protein assignments to control were eliminated.

Table 4.1 Selection of antigenic candidates following mass spectrometry. Data from Scaffold 4 (v4.8.4) was used for peptides coverage. Molecular weight and known cellular location of antigens based on data from Genecards website (https://www.genecards.org).

Candidate	Molecular weight	Peptides coverage	Location
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	36053 Da	85%	Plasma membrane, extracellular, cytoskeleton, nucleus, cytosol, mitochondrion
Nucleophosmin (B23)	32575 Da	64%	Cytoskeleton, nucleus, cytosol, plasma membrane
Inorganic pyrophosphatase 2 (PPA2)	37920 Da	25%	Mitochondria, extracellular
Cathepsin Z (CTSZ)	33868 Da	17%	Plasma membrane, endoplasmic reticulum, lysosome, extracellular
Prohibitin-2	33296 Da	85%	Extracellular, mitochondrion, nucleus, cytosol, plasma membrane
L-lactate dehydrogenase B chain (LDHB)	36638 Da	71%	Cytosol, mitochondrion, plasma membrane
Malate dehydrogenase 2 (MD2)	35503 Da	70%	Extracellular, mitochondrion, cytosol, plasma membrane, nucleus
Malate dehydrogenase 1 (MD1)	36426 Da	56%	Extracellular, mitochondrion, cytosol, cytoskeleton, plasma membrane, nucleus
Protein SEC13	35541 Da	21%	Extracellular, nucleus, endoplasmic reticulum, cytosol, lysosome, Golgi apparatus, plasma membrane.
Cytosolic Fe-S cluster assembly factor (NUBP1)	34534 Da	17%	Plasma membrane, extracellular, cytoskeleton, cytosol
Immunity associated protein 1 (GIMAP1)	34369 Da	14%	Endoplasmic reticulum, Golgi apparatus, plasma membrane
Lys-63-specific deubiquitinase (BRCC3)	36072 Da	12%	Nucleus, cytosol, cytoskeleton, plasma membrane
WD repeat-containing protein 5 (WDR5)	36588 Da	4%	Nucleus, cytoskeleton, cytosol, Extracellular

Figure 4.4

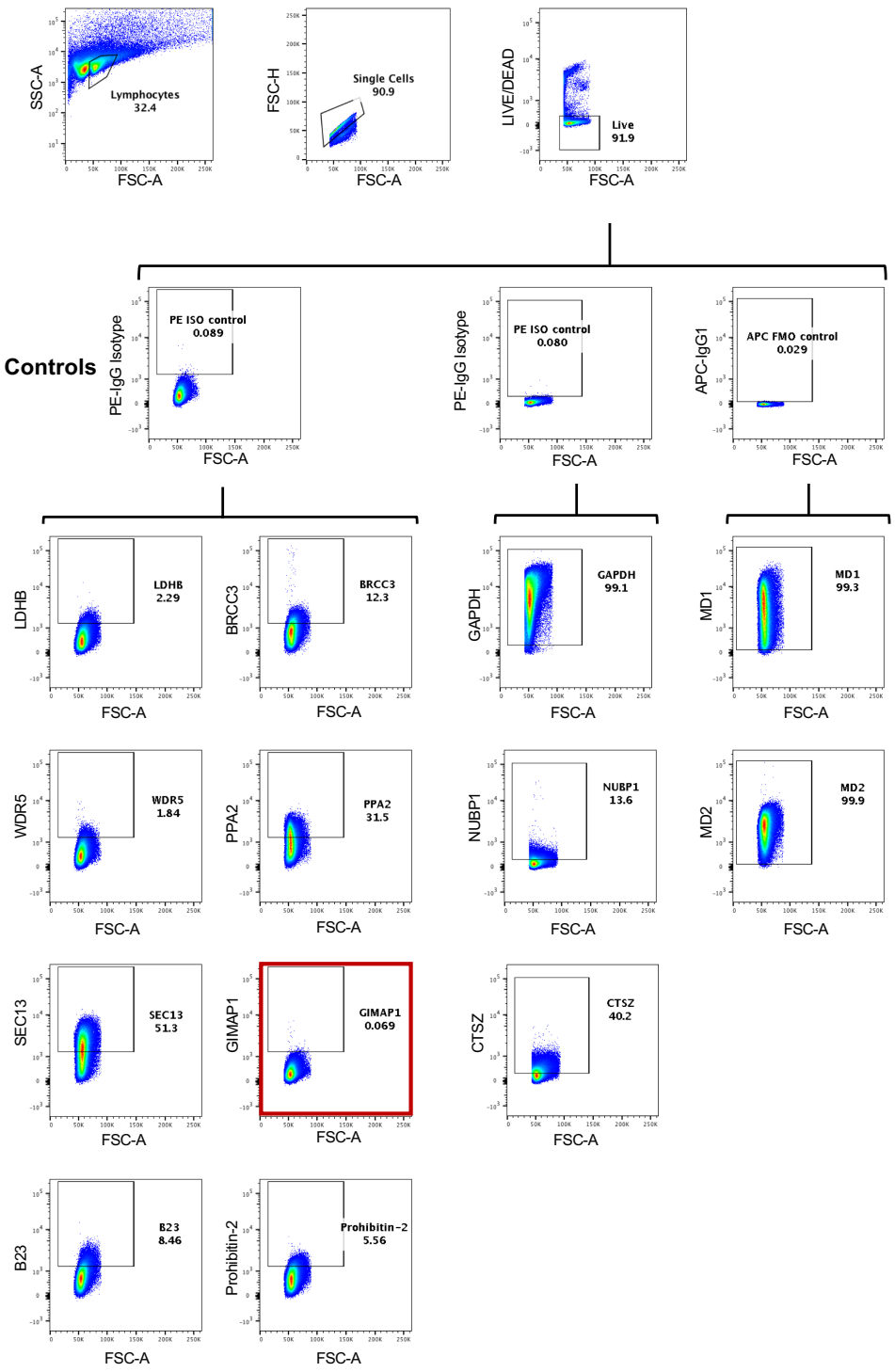

Figure 4.4. Testing of antibody reactivity to antigen by flow cytometry. A tonsil sample was stained with 13 different antibodies. Isotype control and FMO control were used to validate antibody binding to antigen. GIMAP1 antibody did not show any antibody reactivity to antigen (marked in red).

Figure 4.5

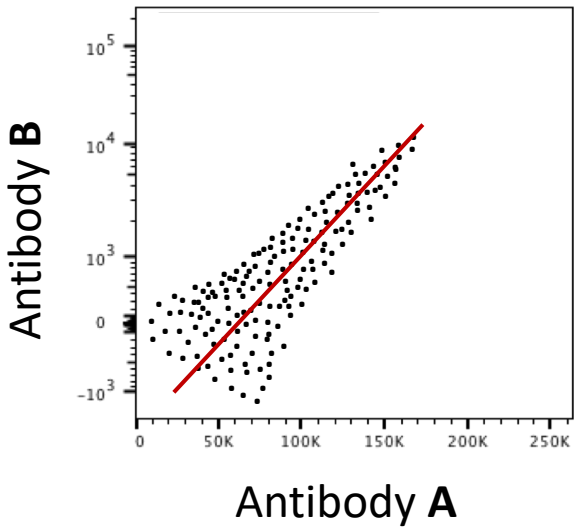

Figure 4.5. Illustration of a flow cytometry plot for the same antigenic expression detected by two antibodies. If two antibodies (A and B) recognised the same antigen, they will bind equivalently and proportionally to cells resulting in a flow plot resembling a hypotenuse triangle or a diagonal line.

Figure 4.6

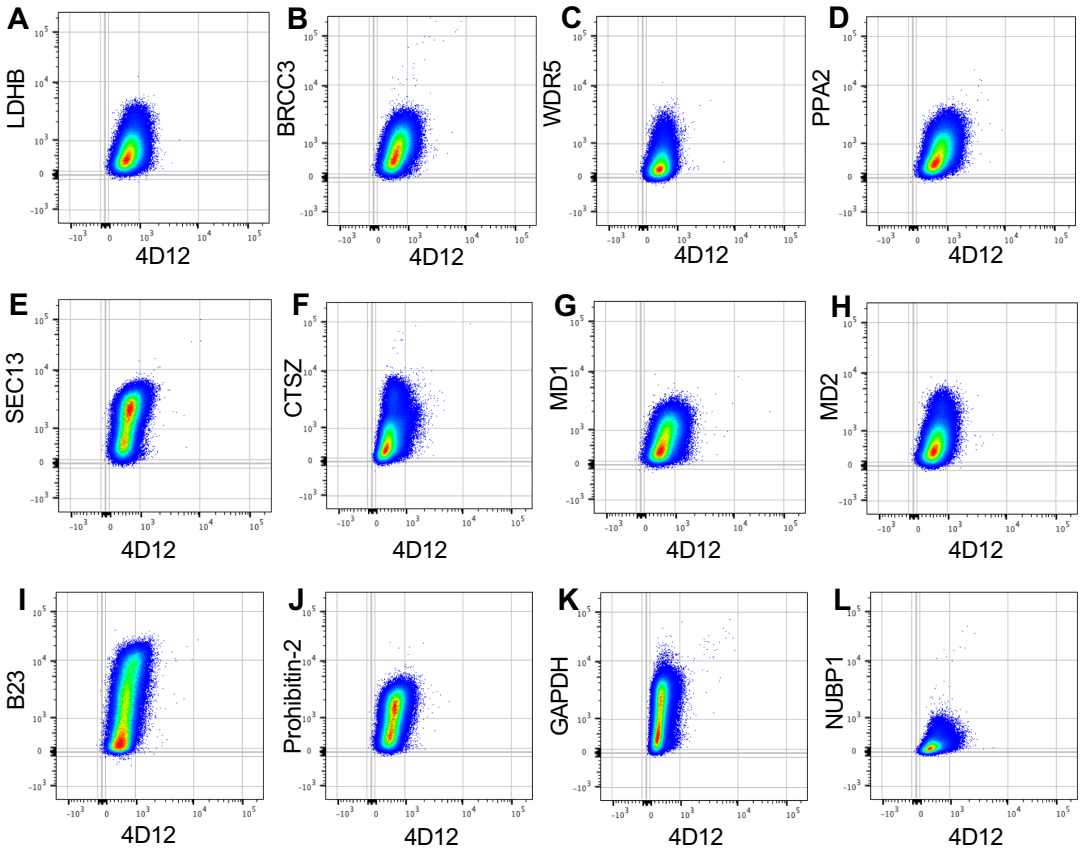

Figure 4.6. Testing for identity between known target antigen and 4D12 antigen in blood sample by flow cytometry (**A**) L-lactate dehydrogenase B chain (**B**) Lys-63-specific deubiquitinase (**C**) WD repeat-containing protein 5 (**D**) Inorganic pyrophosphatase 2 (**E**) Protein SEC13 (**F**) Cathepsin Z (**G**) Malate dehydrogenase 1 (**H**) Malate dehydrogenase 2 (**I**) Nucleophosmin (**J**) Prohibitin-2 (**K**) Glyceraldehyde-3-phosphate dehydrogenase (**L**) Cytosolic Fe-S cluster assembly factor.

Figure 4.7

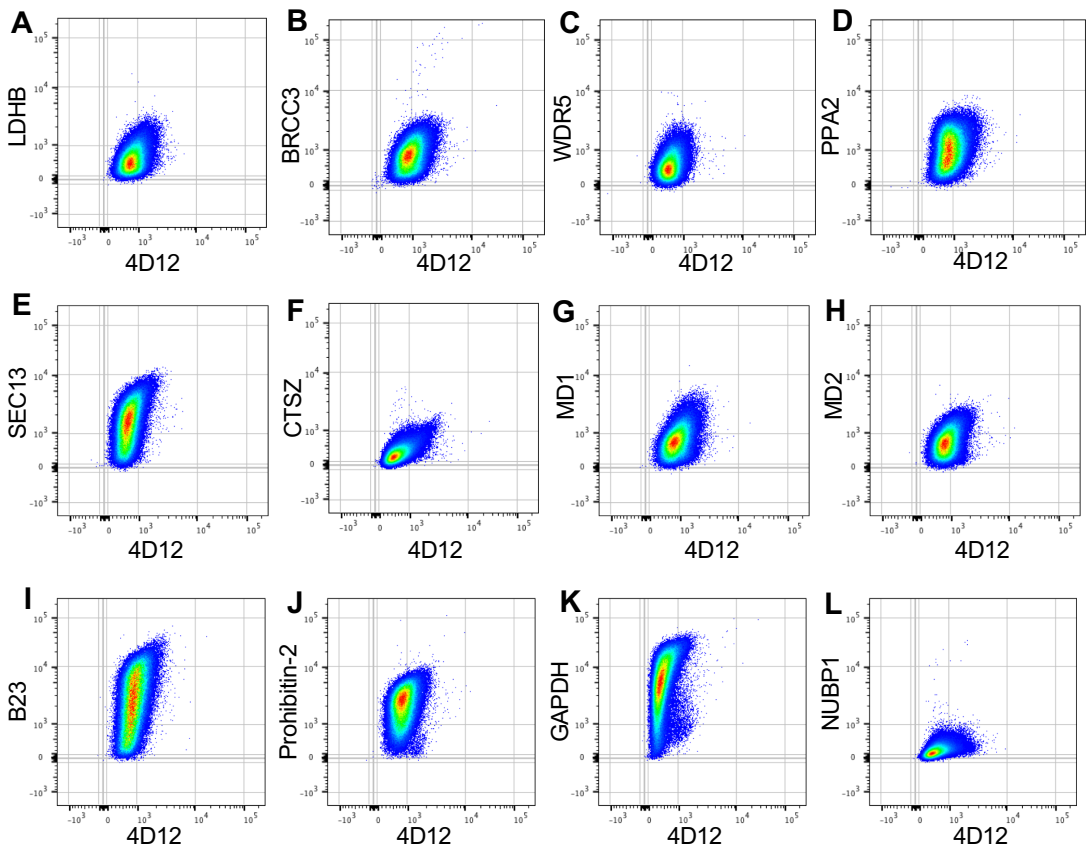

Figure 4.7. Testing for identity between known target antigen and 4D12 antigen in tonsil sample by flow cytometry (**A**) L-lactate dehydrogenase B chain (**B**) Lys-63-specific deubiquitinase (**C**) WD repeat-containing protein 5 (**D**) Inorganic pyrophosphatase 2 (**E**) Protein SEC13 (**F**) Cathepsin Z (**G**) Malate dehydrogenase 1 (**H**) Malate dehydrogenase 2 (**I**) Nucleophosmin (**J**) Prohibitin-2 (**K**) Glyceraldehyde-3-phosphate dehydrogenase (**L**) Cytosolic Fe-S cluster assembly factor.

Chapter 5:
Overview

Overview

This thesis described the distribution of 4D12 antigen on the surface of B cell subsets and in the cytoplasm of all lymphocytes. The histological distribution of the 4D12 antigen did not reflect such broad reactivity observed by intracellular flow cytometry. The inconsistency in the results could be caused by the fixation of the tissue sections, that could have led to the denaturation and damage of antigen. In this case, the marginal zone and germinal centre distribution could be staining artifacts. One the other hand the 35kDa band observed in a western blot was only in B cells and not the control NK cell line. Further analysis of tissues with alternative fixation strategies, and further tissues and cell lines by western blot may help to resolve these contradictions.

The analysis of mass spectrometry and testing for antigen identity by flow cytometry did not successfully identify 4D12 antigen. The outcome of the western blot prior to mass spectrometry suggested a molecular weight of 4D12 antigen to be approximately 35kDa. Future plans for this project could include the use of immunoprecipitation that would be a more sensitive and accurate way to isolate the 4D12 antigen before mass spectrometry to find out the identity of 4D12 antigen.

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